CARDIOVASCULAR ACTIONS OF APELIN RECEPTOR AGONISM DURING RENIN-ANGIOTENSIN SYSTEM ACTIVATION, EXERCISE AND IN PATIENTS WITH CHRONIC STABLE HEART FAILURE

BY

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ABSTRACT

The apelin-apelin receptor (APLNR) system is an important regulator of cardiovascular homeostasis both in health and disease. Principal actions of the apelin-APLNR system are positive inotropism, vasodilatation, diuresis and a potential anti-inflammatory role in vascular tissue.

The significance of this system is highlighted in heart failure and pulmonary hypertension. Preclinical models of these diseases report downregulation of apelin-APLNR, whilst knockout strains develop more severe phenotypes, more rapidly. Moreover treatment with exogenous apelin retards or prevents disease progression.

In man plasma apelin concentrations are reduced in heart failure and vary with disease severity. Initial increases are reported in mild heart failure suggesting a compensatory role, but are depressed in severe heart failure. Limited data profile myocardial APLNR expression in heart failure and in keeping with plasma apelin concentrations, expression is reduced in severe heart failure.

Of interest, the APLNR most closely resembles the angiotensin II type 1 receptor (AT1R), sharing similar tissue expression and sequence homology, but mediates opposing physiological actions. Furthermore, emerging preclinical data support receptor interactions between the APLNR and AT1R that modify their native signalling pathways. It is likely that the apelin-APLNR system serves to antagonise the renin-angiotensin system. Given the established role of angiotensin II, arguably the most important peptide in cardiovascular pathophysiology, any system influencing its actions merits further investigation.

Current clinical studies are limited to 20 minutes infusions and understanding its cardiovascular effects requires more prolonged administration. There are concerns of tachyphylaxis and interaction with the renin-angiotensin-aldosterone system (RAAS**)**, possibly reducing efficacy of APLNR agonism in clinical settings.

In a series of randomised, blinded crossover clinical trials 60 healthy volunteers and 20 patients with chronic stable heart failure were enrolled to assess the effects of (Pyr^{1})apelin-13 infusion at rest, during acute and subacute infusion, exercise and upregulation of the renin-angiotensin system. I have identified that APLNR agonism is unaffected by prevailing levels of angiotensin II activity in local vascular beds and systemic haemodynamic infusions. Furthermore, the efficacy of (Pyr^{1})apelin-13 is retained in healthy volunteers and patients with chronic stable heart failure during acute and subacute infusions. Finally, systemic (Pyr^{1})apelin-13 does not alter exercise performance in healthy individuals.

My findings support a role in targeting the APLNR in chronic heart failure and predict that efficacy will be retained in chronic dosing. Future research directed at other patient groups with ventricular dysfunction is merited, in order to further characterise the utility of this system. These studies are encouraging; however, longer term studies may reveal effects beyond haemodynamic alterations and

examine the effects on cardiac fibrosis and endothelial function. A long acting agonist is required to fully evaluate the role of APLNR signalling in cardiovascular disease.

DEDICATION

To my family.

CONTENTS

ABBREVIATIONS

ACE	Angiotensin-converting enzyme
Ang II	Angiotensin II
ANOVA	Analysis of variance
AP	Alkaline phosphatase
APJ	Apelin receptor
APLNR	Apelin receptor
ApoE	Apolipoprotein E
ARBs	Angiotensin II receptor blockers
AT1R	Angiotensin II type 1 receptor
ATP	Adenine triphosphate
ATRA	All-*trans* retinoic acid
β-blockers	Beta-blockers
β-catenin	Beta-catenin
BSA	Body surface area
Ca^{++}	Calcium ion
CI	Cardiac index
CO	Cardiac output
DBP	Diastolic blood pressure
DCM	Dilated cardiomyopathy
DAG	Diacylglycerol
EDTA	Ethylene diamine tetraacetic acid
EEMeC	Edinburgh Electronic Medical Curriculum
ELISA	Enzyme-linked immunosorbent assay
FGF-2	Fibroblast growth factor-2
GMPG	Good Manufacturing Practice Grade
GPCR	G protein-coupled receptor
IPAH	Idiopathic pulmonary arterial hypertension
IP3	Inositol-1, 4, 5-trisphosphate (IP3)
LDL	Low-density lipoprotein

MAP	Mean arterial pressure
miRNAs	Micro ribonucleic acids
mPTP	Mitochondrial permeability transition pores
NHE	Sodium-hydrogen ion exchanger (Na^+/H^+ exchanger)
NO	Nitric oxide
NYHA	New York Heart Association
PAEC	Pulmonary artery endothelial cells
PAI-1	Plasminogen activator inhibitor type 1
PIP_2	Phosphatidylinositol-4, 5- bisphosphate
PPARγ	Peroxisome Proliferator-Activated Receptor gamma
PVRI	Peripheral vascular resistance index
RAAS	Renin-angiotensin-aldosterone system
RAP	Right atrial pressure
RISK	Reperfusion injury salvage kinases
SBP	Systolic blood pressure
SEM	Standard error of the mean
siRNA	Small interfering ribonucleic acid
SVR	Systemic vascular resistance
T2DM	Type 2 diabetes mellitus
TEB	Thoracic electrical bioimpedance

DECLARATION

This thesis represents research undertaken at the Centre for Cardiovascular Sciences, University of Edinburgh, the Department of Cardiology, Royal Infirmary of Edinburgh and the Division of Experimental Medicine, Imperial College London.

The studies presented in this thesis were supported through a British Heart Foundation Clinical PhD Fellowship Award (FS/09/019). I was personally involved in all vascular assessments and data analysis presented in Chapters 2,3,4,6 and 7, and worked within a research group to undertake a proportion of studies and data analysis for data presented in chapter 5. Chapters 1, 2, 3, 4, 5 and 6 have been published in peer-reviewed journals. I have copyright permission for including the printed journal manuscripts within this thesis.

The thesis has not been accepted in any previous applications for a degree, and all sources of information have been acknowledged. All studies were undertaken in accordance with the regulations of the Lothian or London Riverside Ethics Committee and with World Medical Association's Declaration of Helsinki. The written informed consent of each subject or patient was obtained before entry into the study.

Gareth David Barnes

2015

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I would like to thank all the staff at the Clinical Research Facility, Edinburgh Royal Infirmary. There was no shortage of help from all the staff and in particular Finny Paterson deserves special praise. The Clinical Research Facility is an excellent environment to conduct research. Without volunteers, healthy and patient groups alike, donating their time, no studies would have been possible and I thank them for their part in my research.

In addition to the rich research environment The University of Edinburgh provides, it was a very sociable environment to work in. “The Barn” never failed to provide excellent colleagues, stimulating conversation and help with all aspects of research.

(The “Tour De France” re-make at ESC was a particular highlight). Not all brachial arteries are made equal and more experienced researchers passed on their knowledge freely. In particular I would like to thank Christian Pedersen for his friendship throughout. We started our respective projects at similar times and faced similar challenges along the way. At Imperial College I am especially thankful to Rohini, Sunny and Ola.

Jean Cunningham has worked tirelessly and been invaluable in producing a document that can be submitted. I will always be hugely appreciative and grateful for the time and effort she has spent with me.

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CHAPTER 1

INTRODUCTION:

APELIN AND THE APELIN RECEPTOR SYSTEM; INTERACTION WITH THE RENIN-ANGIOTENSIN SYSTEM AND TRANSLATIONAL PROMISE

Barnes G, Japp AG, Newby DE.
Translational promise of the apelin-APJ system.
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OVERVIEW

The apelin receptor (APLNR) and its ligand, apelin, constitute a relatively new peptidic system with an emerging and important physiological and pathophysiological role that is currently being defined (Figure 1.1). *In vitro* and preclinical models have suggested that the apelin-APLNR system plays an important role in cardiovascular homeostasis as well as fluid balance and metabolism.

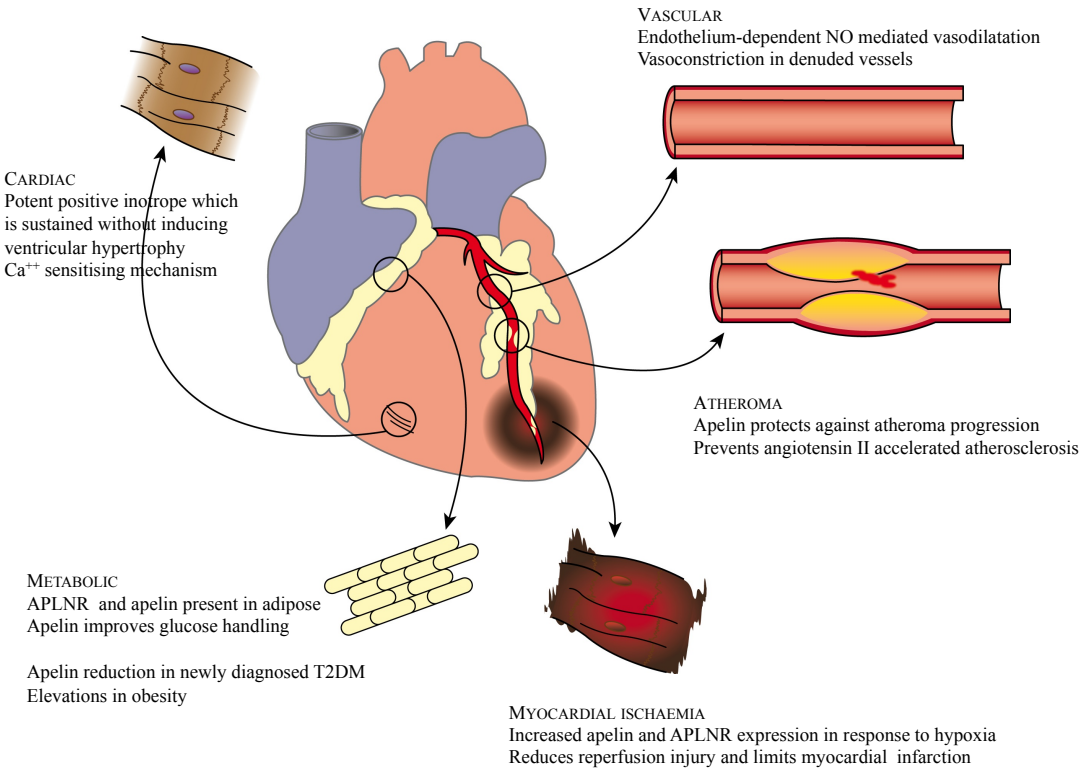

Figure 1.1. Cardiovascular role of the apelin-APLNR system in health and disease.
NO - nitric oxide; Ca^{++} - calcium ion; Ang II - angiotensin II; APLNR - apelin receptor; AP - alkaline phosphatase; T2DM - type 2 diabetes mellitus.

1.1 APELIN AND THE APELIN RECEPTOR

The apelin receptor is a G protein-coupled receptor (GPCR), first identified in 1993 [O'Dowd *et al* 1993]. It is expressed in a wide range of tissues, including the endothelium, myocardium [Hosoya *et al* 2000; Kleinz *et al* 2004; Kleinz *et al* 2005; Farkasfalvi *et al* 2007, Földes *et al 2003*]], adipose tissue [Hosoya *et al* 2000], brain [De Mota *et al* 2000; Medhurst *et al* 2003], renal (Hus Citheral *et al 2008*], gastro-intestinal tract, spleen, adrenal glands, placenta [Medhurst *et al* 2003] and adipose tissues [Boucher et al 2005] (Table 1). The apelin receptor remained orphaned until 1998, when its ligand apelin (APJ receptor Endogenous LigaNd)** was extracted from bovine stomach tissue [Tatemoto *et al* 1998]. It is unclear whether apelin acts on the APLNR in an autocrine, paracrine or hormonal manner, but most evidence supports a paracrine focus of action.

**The APLNR has previous synonyms including AGRL1, APJ, APJR, and FLJ90771; the nomenclature of apelin was based on APJ, however APLNR is the accepted term.

Tissue	Apelin	APLNR
Brain	+	+
Spinal cord	++	+++
Thalamus	+	+
Hypothalamus	+	+
Pituitary	+	+
Cerebellum	+	+
Cerebrum	+	+
Lung	+++	+++
Heart	+	+
Left ventricle	+	+
Right ventricle	+	+
Atria	+++	+++
Kidney	+	+
Glomeruli		+++++
Proximal convoluted tubule		+
Proximal straight tubule (cortex)		+
Proximal straight tubule (medulla)		+
Thick ascending limb (medulla)		+
Thick ascending limb (cortex)		+
Collecting duct (outer medulla)		+
Collecting duct (inner medulla)		+
Placenta	++	+++
Liver	-	+
Vasculature	+	+
Smooth muscle	+	+
Endothelium	+	+
Adrenal	+	+
Spleen	+++	++
Skeletal muscle	+	+
Intestine	+	+
Stomach	+	+
Adipocytes	+	+
Pancreas	+	+

Table 1. Distribution of apelin and APLNR, with sub organ and functional unit data where possible.

Apelin is synthesised as a 77 amino acid prepropeptide that is cleaved into a smaller biologically active fragments. The 36 amino acid peptide, which has the highest binding affinity for the APLNR however shorter isoforms are more potent [Kawamata *et al* 2001]. The prepropeptide contains a number paired basic amino acid sites that are targets for endopeptidases (Figure 1.2). Formation of a pyroglutamate *N* terminus is a post-transcriptional modification that reduces enzyme degradation and therefore preserves activity [Garden *et al 1999*]. The pyroglutamated 13 amino acid, (Pyr^{1})apelin-13 is the most potent and most abundant in cardiac tissue [Maguire *et al* 2009]. Although the main source of plasma apelin is unclear, the cardiac atria [Földes *et al* 2003] are likely to be significant contributors. Tissue locations of apelin are summarised in table 1.

The pathways of apelin metabolism and breakdown are currently obscure, but the mature 77 amino acid peptide contains a number of basic residues that are potential cleavage sites for peptidases. Presently, angiotensin-converting enzyme (ACE) II is the only enzyme known to break down the mature apelin peptide [Vickers *et al* 2002]. Apelin has a brief half-life of under 5 minutes in man, and its cardiovascular actions are short-lived [Japp *et al* 2008].

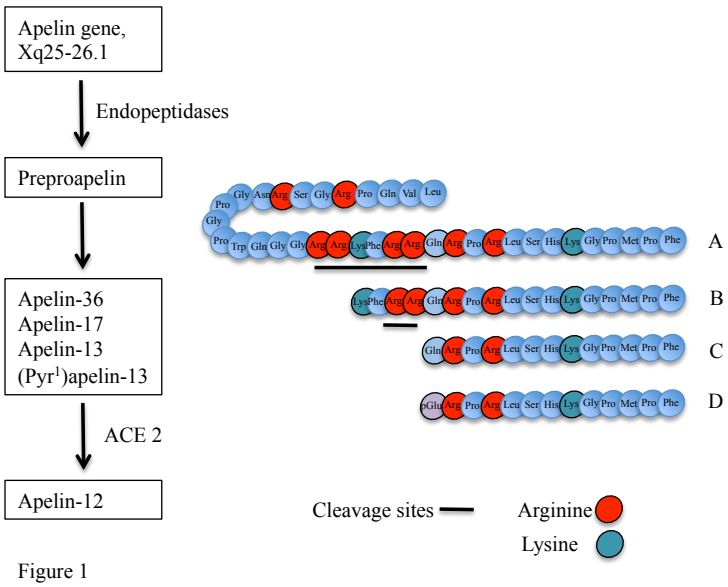

Figure 1
Apelin sythesis and metabolism. Cleavage sites (black underline) in regions with arginine (red) and lysine (green) rich domains. A) Aplein-36. B) Apelin-17 C) Apelin-13. D) (Pyr[1])apelin-13

1.1.2 APLNR SIGNALING

Intracellular signalling cascades are incompletely understood however the APLBR appears to promote signalling through more than one pathway. In Chinese Hamster ovary cells, apelin inhibits forskorlin stimulated cAMP production in a dose dependent fashion [Habata et al 1999], suggesting that APLNR signalling is coupled to Gαi protein. Moreover in cells treated with pertussis toxin, which selectively inhibits Gαi subunits, apelin mediated intracellular signalling is entirely prevented [Masri et all 2002]. Selective pharmacological inhibition of different α subunits identified that it is αi1 and αi2 subunits are coupled to APLNR. [Mesri et all 2006].

However in perfused heart models, pertussis toxin only partially inhibits APLNR mediated positive inotropism [Szodoki et al 2002]. In this model inhibition of

phospholipase C and protein kinase C resulted in greatly reduced effects of APLNR stimulation [Szodoki et al 2002]. Phospholipase C is the major Gαq effector and hydrolyses phosphatidylinositol-4, 5- bisphosphate (PIP_2), generating inositol-1, 4, 5-trisphosphate (IP3) and diacylglycerol (DAG). IP_3 releases cytolsolic calcium stores, whilst DAG activates PKC. Taken together these data suggest that the APLNR receptor can activate intracellular signalling though αi and αq subunits.

1.2 CARDIOVASCULAR ACTIONS OF APELIN

1.2.1 VASCULAR ACTIONS

Apelin receptor stimulation predominantly results in vasodilatation, as demonstrated in *ex vivo* models of animal and human conduit arteries, resistance vessels and veins [Salcedo *et al* 2007; Zhong *et al* 2007b; Maguire *et al* 2009]. Accordingly, intravenous administration in rodents reduces mean arterial pressure [Lee *et al* 2000; Tatemoto *et al* 2001; Cheng *et al* 2003; Ishida *et al* 2004], systemic venous tone [Cheng *et al* 2003] and cardiac preload and afterload [Ashley *et al* 2005]. Apelin-mediated vasodilatation is endothelium-dependent, since vasoconstriction occurs in endothelium-denuded vessels [Katugampola *et al* 2001; Maguire *et al* 2009]. Furthermore, apelin promotes dose-dependent phosphorylation of myosin light chain protein in cultured vascular smooth muscle cells and aortic tissue, both lacking functional endothelium, thereby providing a potential mechanism of vasoconstriction [Hashimoto *et al* 2006; Wang *et al* 2008].

Vasodilatation appears to be mediated through predominantly nitric oxide-dependent pathways: *in vitro* apelin increases nitric oxide synthase transcription [Jia *et al* 2007] and phosphorylation [Zhong *et al* 2007b], while *in vivo* apelin increases plasma nitrate and nitrite concentrations [Tatemoto *et al* 2001]. Furthermore, the inhibition of nitric oxides synthase markedly attenuates both depressor [Tatemoto *et al* 2001] and vasodilator [Japp *et al* 2008] responses. In human mammary arteries and saphenous veins, although not in mesenteric resistance vessels, apelin induces vasodilatation through prostacyclin-dependent pathways [Maguire *et al* 2009]. The vascular effects of apelin in clinical studies mirror those in animal models. Intra-arterial infusion of apelin causes reproducible vasodilatation in human forearm circulation [Japp *et al* 2008]. In this model, vasodilatation to apelin is reduced by two-thirds in the presence of nitric oxide inhibition, but it is unaffected by prostacyclin inhibition [Japp *et al* 2008]. Apelin does not appear to exert *in vivo* vasomotor effects in human dorsal hand veins, but the effects on central capacitance vessels are yet to be studied. In addition to these two studies, data from our own group have demonstrated that apelin is also a coronary vasodilator and, when administered at systemic doses, reduces peripheral vascular resistance [Japp *et al* 2010]. There are no data supporting a vasoconstrictor role for apelin in healthy volunteers or patients. However, given that preclinical studies have demonstrated a need for endothelium to mediate vasodilatation [Katugampola *et al* 2001], it is conceivable that APLNR stimulation may promote vasoconstriction in patients with endothelial dysfunction.

1.2.2 CARDIAC ACTIONS

Apelin is the most potent endogenous inotropic described to date. *In vitro*, exogenous apelin increases contractility at subnanomolar concentrations in atrial strips [Maguire *et al* 2009] and whole rat hearts [Szokodi *et al* 2002], and it increases sarcomere shortening by 140% in isolated cardiomyocytes [Farkasfalvi *et al* 2007]. In healthy rodents, acute apelin infusion increases myocardial contractility, independent of its loading conditions [Ashley *et al* 2005] while, uniquely among current inotropic agents, chronic dosing causes a sustained increase in cardiac output, without inducing left ventricular hypertrophy [Ashley *et al* 2005].

Recent studies have shown that endogenous apelin-APLNR signalling makes an important contribution to maintaining cardiac function. Isolated ventricular myoctyes from both apelin- and APLNR-deficient mice have impaired sarcomeric function resulting in reduced myocyte contractility [Charo *et al* 2009]. Whilst apelin-deficient mice display normal or minimally impaired basal cardiac function, they demonstrate a marked reduction in exercise capacity and maximal oxygen consumption [Charo *et al* 2009]. Furthermore, these knockout rodents manifest progressive cardiac dysfunction from 6 months of age and develop severe heart failure when subjected to chronic pressure overload via surgical aortic banding [Kuba *et al* 2007]. Taken together, these data suggest a critical role for apelin-APLNR signalling in maintaining and augmenting cardiac performance during conditions of cardiovascular stress.

The inotropic actions of apelin are independent of angiotensin II, endothelin, catecholamines and nitric oxide release [Szokodi *et al* 2002]. Although data conflict to some extent, it appears that apelin acts predominantly through mechanisms other than by raising intracellular calcium concentrations (Figure 1.3).

APNLR stimulation in the myocardium activates different pathways that are coupled to different Gα subunits. Through the Gαi subunit, phospholipase C is activated, which in turn generates IP3 leading to protein kinase C activation and increased the activity of sodium-hydrogen ion exchanger (Na^+/H^+ exchanger, NHE). [Szokodi et al 2002]. In addition to activating NHE protein kinase C functions to activate myosin light chain kinase (MLCK), which promotes positive inotropism through increased phosphorylation of sarcomeric proteins. Additionally, through Gαq pathways, extracellular-regulated kinases 1 and 2 (ERK 1/2) is activated through MEK1, which also stimulates NHE. Stimulation of the NHE leads to intracellular alkalinisation and the sensitisation of cardiac myofilaments to intracellular calcium ions [Karmazyn *et al* 1999]. Accordingly, in isolated cardiomyocytes, apelin activates the NHE, thus increasing intracellular pH [Farkasfalvi *et al* 2007]. An additional effect of intracellular alkalisation is activation of the sodium-calcium exchange, which works in reverse to raise intracellular calcium. In these and other studies [Szokodi *et al* 2002] of isolated cardiomyocytes, apelin had no effect on calcium ion transients. Furthermore, isolated cardiomyocytes from APLNR- and apelin-deficient patients with impaired cardiac function show no alteration in calcium ion transients [Charo *et al* 2009]. However, apelin causes a modest increase in the amplitude of the intracellular calcium ion transients in failing rat trabeculae [Dai *et al* 2006] and

isolated cardiomyocytes [Wang *et al* 2008], suggesting the possibility of an additional mechanism involving increased calcium sensitivity.

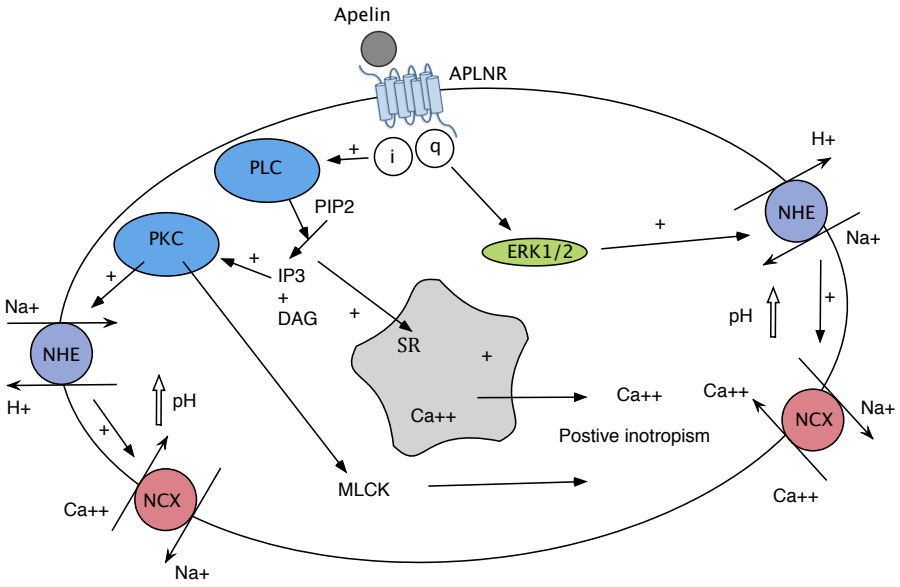

Figure 1.3 Mechanisms of APLNR mediated inotropism. APLNR - apelin receptor; q - Gαq protein; i - Gαi protein; ERK 1/2 extracellular-regulated kinases 1 and 2; PLC - Phospholipase C; SR - sacroplasmic reticulum; Ca^{++} - calcium; NHE - Na^{+}/H^{+} exchanger; NCX - reverse $Na^{+/}Ca^{2+}$ exchanger; PIP2 - phosphatidylinositol 4,5-bisphosphate; IP3 - inositol 3,4,5 trisphosphate; DAG - diacylglycerol.; MLCK - myosin light chain kinase.

1.2.3 EXERCISE PHYSIOLOGY

There are limited data available assessing the contribution of the apelin-APLNR system to exercise physiology. One preclinical study has assessed resting myocardial function and exercise performance in apelin- or APLNR-deficient strains [Charo *et al* 2009]. Both of these knockout rodents had reduced exertion capacity compared to wild type, assessed by endurance time. The contribution of apelin-APLNR in man

during exercise is unclear, as is the effect of exogenous apelin during exercise assessment. However, it is most likely that APLNR agonism will be of benefit in disease with reduced circulating apelin concentrations.

1.2.4 FLUID HOMEOSTASIS

Apelin and the APLNR are present in the kidney and many areas of the brain. High concentrations are found in the supra-paraventricular nuclei and supra-optic nuclei [De Mota *et al* 2000]: regions involved in fluid homeostasis. Here, the synthesis and secretion of apelin appear to be regulated by vasopressin [Reaux-Le Goazigo *et al* 2004]. In turn, intracerebral injection of apelin directly inhibits vasopressin release, leading to a 40% reduction in plasma vasopressin concentrations. In keeping with this notion, apelin has diuretic properties [De Mota *et al* 2004; Hus-Citharel *et al* 2008] that appear to be aquaretic, with little or no apparent increase in sodium excretion [De Mota *et al* 2004]. In man, water loading results in elevated plasma apelin concentrations, while increased plasma osmolality causes plasma apelin concentration to fall; in each case there is a reciprocal change in vasopressin concentration [Azizi *et al* 2008]. Overall, these changes suggest that apelin may counter regulate vasopressin in fluid homeostasis.

1.2.5 GLUCOSE METABOLISM

Apelin is expressed in mouse and human adipose, and *in vitro* adipose tissue synthesises apelin [Boucher *et al* 2005]. Apelin expression tracks plasma insulin concentrations, with fasting reducing adipose expression, which returns to baseline through refeeding [Boucher *et al* 2005]. Exogenous apelin reduces the peak glucose concentration after glucose loading, by increasing glucose turnover [Dray *et al* 2008] through insulin-dependent and independent pathways. Apelin-deficient animal models have reduced insulin sensitivity, and this can be corrected by the administration of exogenous apelin [Yue *et al* 2010]. The effect of exogenous handling in man is currently unknown.

1.3 THE APELIN-APLNR AND RENIN-ANGIOTENSIN SYSTEMS

Among the G protein-coupled receptors, the APLNR displays the closest homology to angiotensin II type 1 receptor (AT1R) at around 50%, predominantly in the transmembrane domains. Furthermore, similar patterns of tissue expression are evident for both receptors [Ashley *et al* 2006]. Physiologically, the actions of the renin-angiotensin system and the apelin-APLNR system are antagonistic, with opposing actions on vascular tone [Gurzu *et al* 2006], blood pressure [Ishida *et al* 2004], atherosclerosis [Chun *et al* 2008] and fluid homeostasis [Hus-Citharel *et al* 2008]. However, there are accumulating data to suggest direct interaction between the two systems that is unique at a cellular level (Figure 1.3).

AT1R-APLNR interaction

The presence of the APLNR modifies the vascular actions of angiotensin II. Myography studies in arteries from diabetic mice, which have reduced APLNR expression, show increased angiotensin II constriction [Zhong *et al* 2007a]. In whole animals, rodents deficient in APLNR and AT1R exhibit elevated baseline mean arterial pressure, supporting the role of the APLNR opposing the renin-angiotensin system [Ishida *et al* 2004]. Furthermore, in these double knockout strains, exaggerated pressor responses to angiotensin II infusion imply that the presence of the APLNR modifies the potency of angiotensin II [Ishida *et al* 2004]. Cellular models have investigated the interaction between these two systems. Human embryonic kidney cells co-transfected with both the AT1R and APLNR have reduced downstream signalling from angiotensin II [Sun *et al* 2011]. In agreement with this finding, knockdown cellular models that reduce APLNR expression with small interfering ribonucleic acid (siRNA) exhibit exaggerated responses to angiotensin II [Chun *et al* 2008]. Therefore, in cellular, myography and whole animal studies, the APLNR antagonises angiotensin II signalling.

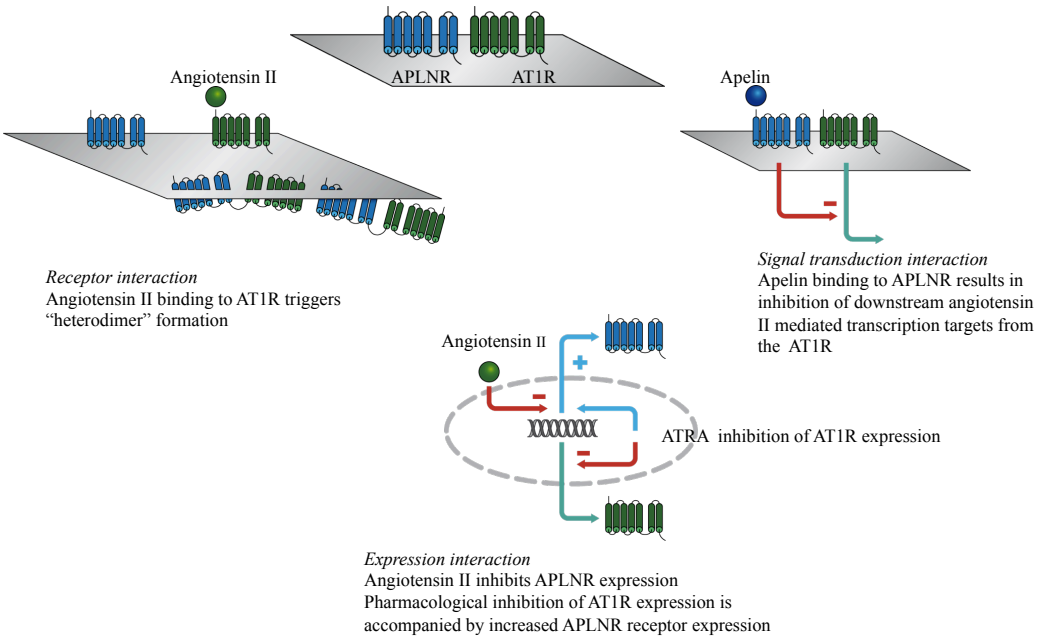

Figure 1.3. Interaction between the apelin-APLNR and renin-angiotensin systems.
APLNR - apelin receptor; AT1R - angiotensin II type 1 receptor; ATRA - all-*trans* retinoic acid.

APLNR and AT1R can form heterodimers [Chun *et al* 2008; Sun *et al* 2011], which is proposed as a significant mechanism for the interaction between these two systems. The AT1R forms heterodimers with several G protein-coupled receptors *in vitro* [AbdAlla *et al* 2000; AbdAlla *et al* 2001; AbdAlla *et al* 2005]. Functionally, the APLNR-formed heterodimer inhibits downstream signalling from the AT1R, which is independent of apelin but promoted by angiotensin II [Chun *et al* 2008; Sun *et al* 2011]. However, heterodimer formation has, as yet, only been observed *in vitro*. Furthermore, studies have been unable to recreate AT1R formation in a range of models [Hansen *et al* 2009], suggesting that this may be a complex interaction requiring specific conditions, and it is unclear if these conditions are recreated in man. It is possible that heterodimers may not form *in vivo,* and this behaviour does not reflect native tissues or has little contribution to tissue metabolism. Additionally there are vast numbers of G protein-coupled receptors, which may be constantly forming dynamic receptor complexes; dissecting the contribution of one interaction in native tissues may be challenging.

The contribution of apelin in the modification of AT1R signalling produces varying results. The inactive APLNR has been reported as key regarding its antagonist role, with apelin preventing heterodimer formation and promoting angiotensin II signals [Sun *et al* 2011]. However, Chun *et al* [2008] reported reduced AT1R signalling in the presence of activated APLNR. Whilst these are similar models, it is possible that alterations to receptor expression and density may exist and explain why differing results are reported.

Counter regulation of gene expression

In addition to cell signalling and receptor interaction, these systems interact at the level of gene expression *in vitro*. In cellular models of maturing adipocytes, blockade of angiotensin II increases apelin expression and peptide secretion [Hung *et al* 2010]. Whole animal models are in keeping with this observation and administration of angiotensin II at subpressor doses reduces cardiac apelin expression, an effect abolished by concurrent AT1R blockage [Iwanaga *et al* 2006]. Furthermore, in hypertensive animal models of heart failure, as the phenotype progresses from left ventricular hypertrophy to ventricular failure, myocardial renin-angiotensin system expression increases, whilst the apelin-APLNR system is downregulated. However treatment with angiotensin II receptor blockers (ARBs) prevents progression to heart failure without altering expression levels for the renin-angiotensin or apelin-APLNR systems [Iwanaga *et al* 2006].

There are further preclinical data supporting opposing expression patterns of the APLNR and AT1R under specific conditions. Mechanical stretch reduces APLNR expression within 24 hours [Szokodi *et al* 2002)] whilst increasing AT1R [Kijima *et al* 1996]. These data suggest that the transcription of the AT1R and APLNR genes expression are closely related.

Transcription of the AT1R is kept under control, at least in part, by the retinoic acid receptor and the retinoid X receptor, both of which are nuclear receptors [Takeda *et al* 2000]. These proteins influence transcription [Bastien and Rochette-Egly 2004], and can be inhibited pharmacologically by all-*trans* retinoic acid (ATRA) treatment.

In vascular smooth muscle cells incubated with ATRA, AT1R expression is reduced [Takeda *et al* 2000]. In animal models of hypertension, with increased renin-angiotensin expression, ATRA also reduces AT1R expression and increases APLNR expression. This is accompanied by a reduction in blood pressure, which appears to be mediated by nitric oxide [Zhong *et al* 2005].

Downstream angiotensin II signalling is modified by the apelin-APLNR system. In whole animals, APLNR signalling opposes profibrotic angiotensin II effects on myocardium, preventing upregulation of plasminogen activator inhibitor type 1 (PAI-1). Furthermore, angiotensin II transcription targets are influenced by APLNR activation. In cultured smooth muscle cells angiotensin II increases PAI-1 promotor activity, transcription and expression, which is inhibited by apelin treatment**.** Consistent with these results, angiotension II induced myocardial fibrosis, which is accompanied with upregulation of profibrotic genes, including PAI-1, is reduced in rodents treatment with apelin [Siddiquee *et al* 2011].

Peptide metabolism

At present, little is known about the circulating source of apelin or its metabolism. It is likely to function in both autocrine and paracrine fashions, with the cardiac atria being likely sources [Földes *et al* 2003]. The only identified enzyme involved in apelin breakdown is angiotensin-converting enzyme II, which hydrolyses both apelin-13 and apelin-36 [Vickers *et al* 2002]. ACE II diverts angiotensin II precursors from the 'classical' renin-angiotensin system, generating angiotensin 1-9 and angiotensin 1-7 from angiotensin I and angiotensin II, respectively. The affinity

of ACE II for angiotensin II is some 400-fold higher than for angiotensin I, suggesting a major role in limiting angiotensin II accumulation and generating angiotensin 1-7 (Vickers *et al* 2002). There is increasing interest in the roles of ACE II and angiotensin 1-7 in cardiovascular physiology and disease. Angiotensin 1-7 acts on the Mas receptor, mediates vasodilatation [Brosnihan *et al* 1996; Sasaki *et al* 2001], is cardioprotective [Loot *et al* 2002] and is antiproliferative [McCollum *et al* 2012], properties very similar to those of apelin. It is likely that the relationship between all of these peptides is highly complex and dynamic, but it is nevertheless conceivable that the apelin-APLNR system is one of a range of endogenous systems that modulates the functions of angiotensin II.

In summary, there may be a reciprocal counter regulation between the apelin-APLNR and renin-angiotensin systems. Given that the apelin-APLNR pathway appears to be inhibited by angiotensin II, this raises the possibility of therapeutic synergism by combining APLNR agonism with the inhibition of the renin-angiotensin system. However, preclinical data highlight the potential for APLNR activation to enhance angiotensin II-mediated AT1R signalling, which is key to understanding *in vivo* in man.

1.4 APELIN-APLNR SYSTEM: TRANSLATIONAL POTENTIAL IN CLINICAL DISEASE

1.4.1 HEART FAILURE

Data from apelin-deficient mice indicate that endogenous apelin activity may help maintain cardiac performance under conditions of cardiovascular stress [Kuba *et al* 2007; Charo *et al* 2009]. However, the apelin-APLNR system appears to undergo downregulation during heart failure. Isolated cardiomyocytes subjected to repeated mechanical stretching, analogous to volume overload in congestive heart failure, exhibit marked downregulation of the APLNR within 24 hours [Szokodi *et al* 2002]. In an *in vivo* rodent model of hypertensive heart disease, the expression of cardiac apelin and the APLNR is increased or maintained at the left ventricular hypertrophy stage, but it declines dramatically with the transition to overt heart failure [Iwanaga *et al* 2006]. In humans, cardiac apelin-APLNR expression is reduced in patients with chronic heart failure secondary to dilated cardiomyopathy [Földes *et al* 2003; Pitkin *et al* 2010]. Interestingly, APLNR expression is preserved in those with an ischaemic aetiology [Földes *et al* 2003], perhaps reflecting the stimulatory effect of local hypoxia on apelin-APLNR expression [Atluri *et al* 2007; Sheikh *et al* 2008; Leeper *et al* 2009; Zeng *et al* 2009].

Several groups have measured plasma apelin concentrations in patients with heart failure. In general, plasma apelin concentrations are maintained for mild to moderate left ventricular failure [Chen *et al* 2003], but they are reduced with severe left ventricular failure [Chen *et al* 2003; Chong *et al* 2006]. The apparent decline in

apelin-APLNR activity, in parallel with deteriorating cardiac performance, suggests a potential role for diminished apelin signalling in the pathophysiology of heart failure. Strategies to augment apelin signalling may therefore help to retard the progression of heart failure. Significantly, in an isoproterenol-induced model of heart failure, left ventricular dysfunction is partially rescued by co-administration of apelin [Jia *et al* 2006]. Tentative evidence indicating that alterations in endogenous apelin-APLNR activity may modulate the progression of heart failure was provided in a cohort of patients with dilated cardiomyopathy [Sarzani *et al* 2007]. APLNR polymorphisms have been identified in a cohort of patients with heart failure. Genotyping identified two single nucleotide polymorphism, G212A and A445C. In patients with heart failure, presence of at least one A allele was associated with slower disease progress. The frequency of alleles was similar in healthy controls and patients with heart failure suggesting a modifying role rather than causal. [Sarzani *et al* 2007].

The apelin-APLNR system is upregulated in response to device implantation, which may be mechanistic for the improvement in myocardial function. The APLNR is the most upregulated gene in response to left ventricular assist device implantation [Chen *et al* 2003] whilst plasma apelin concentrations increase following biventricular pacemaker insertion [Francia *et al* 2007].

The unique haemodynamic profile of apelin in preclinical models suggests potential therapeutic utility in patients with established heart failure. Exogenous apelin potently enhances myocardial contractility, without inducing left ventricular

hypertrophy [Ashley *et al* 2005], and achieves this while simultaneously reducing ventricular preload and afterload [Berry *et al* 2004]. Crucially, the beneficial effects of apelin on cardiac contractility and loading conditions are maintained in preclinical models of heart failure. *In vitro*, apelin increases contractility in failing myocardium to the same [Farkasfalvi *et al* 2007] or greater [Dai *et al* 2006] extent as normal myocardium. *In vivo*, acute apelin infusion restores ejection fraction, increases cardiac output and reduces left ventricular end-diastolic pressure in rats with chronic heart failure [Berry *et al* 2004; Atluri *et al* 2007]. Thus, irrespective of alterations in receptor expression, these studies confirm that APLNR signalling capacity is not exhausted by exogenous apelin in established heart failure, an essential prerequisite for therapeutic strategies employing APLNR agonism.

Preliminary data from clinical studies are encouraging. We have demonstrated that the local vascular and systemic haemodynamic effects of acute apelin infusion, including a rise in cardiac output, are preserved in patients with chronic stable heart failure [Japp *et al* 2010]. Importantly, these patients continued to receive currently available optimal pharmacological treatment, suggesting that the effects of apelin were additional to established heart failure treatments. In particular, all but one of the patients received treatment with an ACE inhibitor or an ARB. Preclinical data report direct interactions between the renin-angiotensin and apelin-APLNR systems, raising the possibility that some of apelin's actions are mediated through antagonism of angiotensin II [Chun *et al* 2008; Siddiquee *et al* 2011]. Nonetheless, our findings imply a role for apelin that is independent of angiotensin II signalling pathways, and

they further suggest the potential for pharmacological synergism through combined APLNR agonism and renin-angiotensin system inhibition.

One further area of translational potential in heart failure is utilising apelin as a biomarker (Table 1.1) [Chen *et al* 2003; Földes *et al* 2003; Chong *et al* 2006; Goetze *et al* 2006; Francia *et al* 2007; Miettinen *et al* 2007]. As discussed above, plasma apelin concentrations appear to decrease in patients with advanced heart failure, and this process may coincide with a decrease in cardiac performance [Chen *et al* 2003; Földes *et al* 2003; Chong *et al* 2006]. The largest study, (n >200) by Chong *et al* [2006], reported a reduction in plasma apelin concentrations in patients with heart failure, across all classes. Whilst there was preponderance towards more severe heart failure, with the majority in New York Heart Association (NYHA) class III or IV, no relationship between severity and plasma apelin concentration was identified. Conversely, Chen *et al* [2003] proposed that plasma apelin was dynamic in heart failure, with an increase in plasma apelin concentration reported in mild heart failure, which reduced to baseline levels in individuals with severe heart failure [Chen *et al* 2003].

Although studies have reported conflicting findings, some discrepancies are likely to be explained by differences in patient populations. Perhaps more importantly, there are significant concerns over the currently available assays for apelin and how these have been used. At present there are no assays available to detect all isoforms, such as (Pyr^{1})apelin-13, which is the most potent and abundant isoform in cardiac tissue [Maguire *et al* 2009]. Equally, there is an apparent lack of sensitivity and marked

variations in measured concentrations, with around a 40-fold discrepancy (90-3580 pg/mL) [Chen *et al* 2003; Földes *et al* 2003] being reported among healthy control populations. However, this is likely to reflect insufficient extraction in some studies, resulting in non-specific binding and reporting falsely elevated plasma concentrations. Interpretation is further complicated by an incomplete understanding of factors regulating the synthesis, post-translational processing and metabolism of apelin.

The therapeutic potential of APLNR agonism is an exciting area for translational research in heart failure. Further studies in animal models with predictable progression to heart failure should determine the ability of APLNR agonism to prevent or delay the onset of a decline in cardiac performance. Detailed clinical research is currently limited by the lack of long acting, orally active therapeutic agents. While long term APLNR agonism may have therapeutic potential in patients with chronic stable heart failure, chronic dosing is not practicable or deliverable in the current context of the parenteral administration of short acting apelin peptide. The advent of oral APLNR agonists will permit the further exploration of potential interactions between the renin-angiotensin and apelin-APLNR systems, which in turn will help to clarify the pathogenic significance of altered apelin signalling in heart failure and the potential for therapeutic synergism with combined APLNR agonism and renin-angiotensin inhibition. In addition, the effects of sustained APLNR agonism on cardiac contractility and systemic haemodynamics require characterisation and are an essential prerequisite for clinical trials in patients with acute decompensated or chronic heart failure. Finally, longitudinal studies of plasma

apelin concentrations in patients with chronic heart failure will help to determine the utility of apelin as a novel biomarker. However, such studies are dependent on the development of an assay that identifies all of the major apelin isoforms.

1.4.2 VASCULAR DISEASE

Apelin appears to have beneficial effects on vascular health. In diabetic mice, it increases vascular nitric oxide generation and reverses endothelial dysfunction [Zhong *et al* 2007b; Zeng *et al* 2009]. In Apolipoprotein E (ApoE)-deficient mice, apelin infusion inhibits atherogenesis and completely abrogates angiotensin II accelerated atherosclerosis: an effect that is independent of blood pressure [Chun *et al* 2008]. Indeed, double knockout mice, deficient in both apelin and ApoE, have accelerated atherosclerosis in comparison with isolated ApoE-deficient mice. These data imply an important antiatherogenic role for endogenous apelin, as well as a potential benefit from exogenous apelin in atherosclerosis [Zhang *et al* 2006; Chun *et al* 2008]. In contrast, one group has reported that the double knockout of the APLNR and ApoE reduced atherosclerotic lesion formation [Hashimoto *et al* 2007], suggesting that the APLNR is required for the progression of atherosclerosis. These contrasting findings are difficult to reconcile, although it is notable that these studies used very different feeding regimes. Future studies exploring the effects of pharmacological APLNR antagonism in ApoE-deficient mice may help to clarify the situation and reveal if there are off-target effects in one or other of the models.

Apelin treatment has reduced aneurysm formation by almost 50% in a mouse model of elastase-induced abdominal aortic aneurysm formation [Leeper *et al* 2009]. Corresponding reductions of macrophage infiltration within the arterial wall, and the local expression of a macrophage colony-stimulating factor (with strong trends towards reductions in tumour necrosis factor α, interleukin 6 and other proinflammatory cytokines), suggest that the predominant mechanism by which this occurs is a direct anti-inflammatory effect within the vessel wall.

The therapeutic potential of APLNR agonism in human vascular disease has yet to be explored. Apelin causes nitric oxide-mediated vasodilatation *in vivo* in forearm resistance vessels of healthy subjects [Japp *et al* 2008], but is not yet known whether it reverses the endothelial dysfunction seen in pathophysiological states such as diabetes mellitus and hypercholesterolaemia. However, in humans, apelin concentrations are reduced in individuals with dyslipidaemia [Tasci *et al* 2007] and coronary artery disease [Li *et al* 2008]. Furthermore, reductions in low-density lipoprotein (LDL) cholesterol concentrations as a result of dietary or statin intervention are associated with an increase in plasma apelin concentration [Tasci *et al* 2009]. Given the promising preclinical data, the potential role of APLNR agonism in preventing human vascular disease now merits detailed investigation. However, while studies have shown a predominant vasodilator action, vasoconstriction has been seen in vessels denuded of endothelium [Maguire *et al* 2009]. This is common with other vasodilators, such as acetylcholine, and it may have implications for the effects of apelin in patients with vascular

disease. Contrary to this possibility, we have recently shown that vasodilatation to apelin is preserved in patients with heart failure [Japp *et al* 2010].

APNLR polymorphisms have been identified in the 212 loci in patients with cornonary artery disease. Whilst with the frequency of 212 polymorphisms similar in coronary artery disease and healthy populations, in patients with coronary artery disease and hypertension, the GG genotype is associated with hypertension, which may suggest a modifying role in this group [Falcone *et al.* 2012]. The biological function and significance of 212 polymorphisms is unclear at present, however this loci may be represent an important modifier role in cardiovascular disease.

1.4.3 MYOCARDIAL INFARCTION AND ISCHAEMIA-REPERFUSION INJURY

Different models of myocardial ischaemia have been used to assess changes in apelin expression, with hypoxia serving as a key stimulus. There is a time-dependent upregulation of apelin in cardiomyocytes maintained at low oxygen tensions under the control of hypoxia inducible factor 1α [Ronkainen *et al* 2007]. In Langendorff models of myocardial infarction and ischaemia-reperfusion, both APLNR and apelin are upregulated [Kleinz and Baxter 2008; Zeng *et al* 2009], especially at the watershed areas of infarct zones [Sheikh *et al* 2008]. This upregulation has been reported to return rapidly to baseline following reperfusion [Kleinz and Baxter 2008] or persist for up to 12 weeks [Sheikh *et al* 2008]. Determining the factors involved in maintaining upregulation is important, as this could serve to prolong the beneficial actions for apelin and may be of therapeutic benefit.

While it is plausible that the main purpose of apelin-APLNR upregulation is to prevent haemodynamic compromise by enhancing coronary blood flow and contractility, there is evidence that apelin acts as a cardioprotective agent to reduce the extent of infarction [Simpkin *et al* 2007]. Restoration of blood flow is a critical step in the resolution of myocardial infarction, but reperfusion does cause further damage to the myocardium and vasculature through ischaemic-reperfusion injury. The factors involved in mediating this injury include adenine triphosphate (ATP) depletion and mitochondrial dysfunction, reactive oxygen species and inflammation. One of the key mechanisms that protects against this injury is the activation of the salvage kinases, notably the reperfusion injury salvage kinases (RISK). This pro-survival pathway reduces ischaemia-reperfusion injury by preserving mitochondrial function. Apelin increases both the phosphorylation and activity of key components within the RISK pathway [Smith *et al* 2007]. One of the hallmarks of mitochondrial dysfunction is the opening of mitochondrial permeability transition pores (mPTP), causing loss of membrane integrity and energy production. Apelin delays mPTP opening, preserves cell structures and reduces mitochondrial damage [Simpkin *et al* 2007].

In the presence of RISK pathway inhibitors, apelin continues to have cardioprotective effects [Kleinz and Baxter 2008], suggesting alternative mechanisms of action. Indeed, during ischaemia-reperfusion injury, apelin increases endothelial nitric oxide synthase expression and reduces oxidative stress by preventing superoxide dismutase degradation [Zeng *et al* 2009]. Apelin also greatly

reduces the generation of reactive oxygen species as a result of catecholamine-induced myocardial damage [Jia *et al* 2006]. Functionally, these protective effects translate into reductions in tissue damage markers, decreased infarct volume and the preservation of ventricular function [Zeng *et al* 2009].

These studies highlight apelin as an agent that protects against myocardial injury, although data are presently limited to preclinical studies. Obvious boundaries make preconditioning agents difficult to implement in clinical practice, but APLNR agonism may have a therapeutic role in patients following an acute myocardial infarction, with potential benefits in both restoring or maintaining left ventricular function and improving survival.

1.4.4 HYPERTENSION

Apelin receptor agonists represent a potentially novel class of antihypertensive agents. In preclinical models, exogenous apelin administration lowers blood pressure through peripheral vasodilatation. The depressor effect of apelin is greatly enhanced in hypertensive animals compared with normotensive controls [Lee *et al* 2005]. The apelin-APLNR system has been investigated in the spontaneously hypertensive rat [Zhang *et al* 2006], in which the apelin-APLNR system is downregulated in ventricular and aortic tissues as hypertension develops suggesting a role in maintaining normal blood pressure. When these hypertensive rodents are randomised to an exercise regime or a sedentary lifestyle, there is upregulation of apelin-APLNR, along with a corresponding reduction in blood pressure [Zhang *et al* 2006]. However,

a mechanistic role for the apelin-APLNR system in this context is not clear, and further investigation is required to tease out any causative role.

Pharmacological inhibition of AT1R expression in the spontaneously hypertensive rat results in a fall in blood pressure and an increase in apelin-APLNR expression. The increase in apelin-APLNR expression may contribute significantly to the hypotensive effect observed [Zhong *et al* 2005]. Given the apparent suppression of APLNR expression by angiotensin II, the antihypertensive effect of APLNR agonism may be enhanced by concomitant treatment with agents that inhibit the renin-angiotensin system. In our preliminary clinical studies, systemic apelin infusion had a modest blood pressure-lowering effect in normotensive middle-aged subjects [Japp *et al* 2008].

1.4.5 IDIOPATHIC PULMONARY ARTERIAL HYPERTENSION

The apelin-APLNR system is downregulated in preclinical models of pulmonary hypertension. In monocrotaline models, both apelin and APLNR expression are reduced in the right ventricle [Falão-Pires *et al* 2009]. Hypoxic models report an initial increase in right ventricular apelin expression of tissue concentration [Chandra *et al* 2011; Drake *et al* 2011], which is greatly reduced as right heart failure develops [Drake *et al* 2011]. Furthermore, therapies that prevent pulmonary hypertension in these animal models are associated with increased apelin expression supporting a key role for the apelin-APLNR system in pulmonary hypertension [Drake *et al* 2011]. In hypoxic models of pulmonary hypertension, rodents that are apelin-deficient develop

more severe pulmonary hypertension than wild types, with the pulmonary vasculature demonstrating obliteration of small calibre arteries within the lung [Chandra *et al* 2011]. Adminstration of exogenous apelin can either prevent or rescue the pulmonary hypertension phenotype in this model [Dai *et al* 2006].

In cultured human pulmonary artery endothelial cells (PAEC) harvested from patients with idiopathic pulmonary arterial hypertension (IPAH) there is reduced apelin expression and increased smooth muscle proliferation. This proliferation can be inhibited with exogenous apelin, which appears to be mediated through signalling involving peroxisome proliferator-activated receptor gamma (PPARγ) and beta (β)-catenin complexes [Alastalo *et al* 2011]. Furthermore, apelin inhibits fibroblast growth factor-2 (FGF-2) regulation in PAECs from patients with IPAH, in a mechanism involving micro ribonucleic acids (miRNAs) (424 and 503). The importance of miRNAs 424 and 503 is highlighted by their ability to rescue and prevent pulmonary hypertension in animal models [Kim *et al* 2012].

Human and clinical data are limited, but evidence suggests that apelin may be pathophysiological and represents a therapeutic target. Lung tissue from patients with IPAH has reduced apelin concentration (Kim *et al* 2011) and plasma apelin concentration is reduced in patients with IPAH [Goetze *et al* 2006; Andersen *et al* 2009; Kim *et al* 2011], with one study reporting levels lower than those observed in left ventricular dysfunction.

The apelin-APLNR system may be of critical importance in IPAH, with both pathophysiological and therapeutic potential. There is no disease modifying therapy available to treat patients with IPAH, however data from these preclinical studies

would support a role for the apelin-APLNR system in reversing vascular remodelling, whilst augmenting right ventricular function.

1.4.6 METABOLIC SYNDROME

Apelin is produced by adipose tissue and influences glucose and lipid metabolism as an adipocytokine. Apelin-deficient animal models exhibit reduced insulin sensitivity, which can be corrected by the administration of exogenous apelin [Yue *et al* 2010]. Conversely, exogenous apelin reduces peak plasma glucose concentration following a glucose load by increasing glucose turnover [Dray *et al* 2008], and this effect is preserved in insulin-resistant animal strains [Dray *et al* 2008]. The exact cellular mechanisms leading to increased glucose uptake are not understood completely. Apelin increases glucose uptake through the phosphorylation of insulin-dependent pathway components, such as Akt, although increased glucose uptake is still observed in the presence of the inhibition of this pathway, suggesting that the pathway is both insulin-dependent and insulin-independent.

In preclinical studies, apelin alters feeding habits. In rats with diet-induced diabetes, exogenous apelin decreases food and water intake, but it has no effect on rodents receiving a normal diet [Clarke *et al* 2009].

The effect of exogenous apelin on glucose handling in humans is currently unknown. However, increases in plasma concentrations have been seen during oral glucose tolerance tests and in healthy individuals and those with type 2 diabetes mellitus (T2DM) [Li *et al* 2006]. Interestingly, plasma apelin concentrations are reduced in

patients with newly diagnosed T2DM [Erdem *et al* 2008] but increased in obese non-diabetic individuals [Boucher *et al* 2005]. This may suggest that the initial increases in apelin seen in obesity serve to delay the development of T2DM by preserving glycaemic control.

APLNR agonism may offer therapeutic potential for the treatment of metabolic syndrome and diabetes mellitus, and it may provide benefits beyond glycaemic control in view of the interaction with the renin-angiotensin system. All functional data are limited to preclinical models, and while the observational data are in keeping with a role in glucose homeostasis, the effect of APLNR agonism needs to be assessed *in vivo* in humans.

1.4.7 APLNR AS A THERAPUETIC TARGET

Conformational changes in receptor structure influences intracellular signalling cascades. Agonist binding, in addition to triggering phosphorylation of GPCR kinases, additionally promotes β-arrestin recruitment and binding to the receptor, which functions to inhibit signal. Additionally, β-arresting binding results in receptor endocytosis via clarthrin-dependant mechanism, which further inhibits agonist responses. Balanced agonists stimulate both G-protein effects and β-arrestin pathway, whereas biased agonists can direct signalling preferentially. [Shukla *et al* 2014]

There are concerns regarding tachyphalaxis, which may limit the therapeutic potential of APLNR agonism. In cellular models, upon agonist binding the APLNR

localizes rapidly to the perinuclear compartment in clathrin and β-arrestin pathways. Interestingly shorter, more potent forms of apelin appear to trigger more internalization. [El Messari *et at* 2004]

There is interest in developing agonists that are biased towards the desired effects of APLNR signalling. ML233 is a small molecule APLNR agonist that has been shown to stimulate G protein pathways, however also results in β-arrestin internalization, and therefore may be of limited utility in further exploring the therapeutic potential of the APLNR. [Khan *et al* 2011]

MM07 is a synthetic molecule that appears to function as a biased agonist for APLNR. In cellular models, β-arrestin mediated internalsation assays MM07 results in reduced receptor internalization, being around two orders of magnitude less potent than (Pyr1)apelin-13. The cardiac vascular effects have been assessed in animal and human models. In whole animal models MM07 results in positive inotropism with increased cardiac output of around 20%, but with out inducing hypotension or reflex tachycardia. This effect is similar to that of other commonly used inotropes, such as dobutamine or milrinone. In man MM07 mediates vasodilation in local forearm studies that is two fold that of (Pyr1)apelin-13, dose dependent and retains efficacy over repeated doses. [Brame *et al* 2015]

Development of biased agonists may unlock the therapeutic potential of the APLNR receptor in cardiovascular disease and will need further evaluation in patient cohorts.

1.4.7 CONCLUSIONS

Both preclinical and emerging clinical studies suggest an important role for apelin in health and disease. However, its contribution to the pathophysiology of cardiovascular diseases needs to be more fully characterised and better defined. Therapeutic manipulation of the apelin-APLNR system represents a novel and potentially exciting therapeutic target, especially in heart failure, vascular disease, myocardial ischaemia and metabolic syndrome.

1.5 STUDY AIMS

The aim of this work is to understand and characterise the cardiovascular actions of the apelin-APLNR system. To date, we have investigated regional and systemic cardiovascular responses to apelin in healthy volunteers and patients with stable heart failure during brief infusions, at rest and with no modification to the renin-angiotensin system.

There is growing evidence of an interaction between the apelin-APLNR and renin-angiotensin systems from *in vitro* and preclinical data. We wish to assess any physiologically relevant relationship in the human vasculature under conditions of renin-angiotensin II activation. Therefore, we need to evaluate whether apelin-APLNR signalling retains its efficacy in the presence of elevated angiotensin II as this is critical in understanding any therapeutic role; many potential applications of apelin will be in diseases that have increased renin-angiotensin II activity.

Furthermore, characterising the effect of prolonged APLNR agonism is essential. The APLNR internalises rapidly, may exhibit tachyphylaxis and is downregulated in heart failure. Taken together, this may suggest that the APLNR may become saturated and the effects short-lived. This would potentially limit its utility as a therapeutic target in heart failure.

Finally, deficiencies in the apelin-APLNR system result in reduced exercise performance. We wish to asses the effect of exogenous apelin during exercise, in order to ascertain whether pharmacological activation of the APLNR during exercise is performance enhancing.

1.6 HYPOTHESES

These following hypotheses will be addressed in this respect:

1. Subacute upregulation of the renin-angiotensin system will reduce the activity of APLNR agonism in local vasculature and systemic circulation by reducing APLNR density in target tissues (Chapter 3).

2. Local and system cardiovascular actions of (Pyr^{1})apelin-13 will be reduced during acute angiotensin II elevation, through reduced availability of the APLNR, and this may even potentiate AT1R signalling (Chapter 4).

3. Brief systemic (Pyr^{1})apelin-13 infusion will mediate an increased cardiac index and reduced mean arterial pressure and systemic vascular resistance in patients with chronic stable heart failure (Chapter 5).

4. Prolonged infusion of (Pyr^{1})apelin-13 will result in sustained systemic cardiovascular actions in healthy volunteers and patients with chronic stable heart failure (Chapter 6).

5. Infusion of (Pyr^{1})apelin-13 will increase exercise performance in healthy volunteers (Chapter 7).

CHAPTER 2

GENERAL

2.1 RECRUITMENT

Healthy volunteers

Subjects were recruited through a local advertisement placed at the campuses of both the University of London and Imperial College London and notifications on the Edinburgh Electronic Medical Curriculum (EEMeC). Permission for medical student recruitment was approved following an application to the University of Edinburgh.

Patients with chronic stable heart failure

Potential participants were identified from the Royal Infirmary of Edinburgh heart failure database and screening of outpatients' clinical notes. An information sheet was sent to all patients fulfilling the inclusion and exclusion criteria. Prior to any contact being made with potential participants, approval was obtained from their regular Consultant Cardiologist.

Patients with chronic stable heart failure were required to have fractional shortening <20% and left ventricular ejection fraction <30%, on an echocardiogram within 6 months of enrolment, and NYHA class II-IV symptoms. Patients were maintained on maximally tolerated doses of evidenced-based therapies, including renin-angiotensin and beta-adrenergic inhibitor therapy, although they abstained from their regular medications on the morning of study.

Patients were excluded if they were involved in any other medical trial or had severe and significant co-morbidity, haemodynamically significant aortic stenosis,

malignant arrhythmias, severe hypertension (systolic blood pressure (SBP) >190 mmHg), a pacemaker in situ or women of child-bearing potential.

2.1.1 ETHICS

All studies were conducted following the approval of the Lothian Research Committee (REC 09/S1101/4 and REC 10/S1101/18) and the London Riverside Committee (REC 11/LO/2063) and were in accordance with the World Medical Association's Declaration of Helsinki. Prior to commencing the studies, the participants received a patient information sheet, and written informed consent was obtained.

2.1.2 REGISTRATION

All studies were registered on open access *clinicaltrials.gov* under the following identifiers:

NCT00901719; http://clinicaltrials.gov/ct2/show/NCT00901719

NCT00901888; http://clinicaltrials.gov/ct2/show/NCT00901888

NCT01049646; http://clinicaltrials.gov/ct2/show/NCT01049646

NCT01179061; http://clinicaltrials.gov/ct2/show/NCT01179061

NCT01590108: http://clinicaltrials.gov/ct2/show/NCT01590108.

2.2 UPREGULATION OF THE RENIN-ANGIOTENSIN SYSTEM

2.2.1 ENDOGENOUS, SUBACUTE

The renin-angiotensin system is dormant in healthy individuals consuming a diet replete in sodium. However, this can be activated by consuming a sodium deplete diet [Newby *et al* 1997b]**.** The subjects attended twice at least one week apart, having been randomised in a single blind crossover design to a sodium depleted or normal sodium replete diet, as previously described. Briefly, for sodium depletion, subjects were asked to adhere to a diet containing <12 mmol of sodium per day for 3 days prior to the study visit (see Appendix p186). To ensure prompt sodium depletion, the subjects were given a single oral dose of furosemide (20 mg) on day one of the diet. For all sodium depletion studies, urine was collected from each individual 24 hours before each visit, in order to assess sodium excretion.

2.2.2 EXOGENOUS, ACUTE

Exogenous angiotensin II (Clinalfa AG, Läufelfingen, Switzerland) was infused intra-arterially in local studies or through a 17-gauge venous cannula in the antecubital fossa in systemic studies.

2.3 ASSESSMENT OF LOCAL VASCULAR FUNCTION

2.3.1 VENOUS OCCLUSION PLETHYSMOGRAPHY

The vasomotor function of arteries can be assessed as a measure of endothelial function. Local intra-arterial administration combined with venous occlusion plethysmography [Wilkinson and Webb 2001] in the forearm, provides a method of directly assessing resistance vessel function without the need for systemic administration of investigational agents that can induce counter regulatory neurohormonal responses. Furthermore, this technique assesses vasomotor response in the presence of endogenous physiological regulators of vascular tone and function, providing a true response of vasomotor activity *in vivo* [Webb 1995].

Venous occlusion plethysmography detects flow changes by assessing resistance in strain gauges placed around circumference of the forearm (Figure 2.1). Venous outflow is stopped abruptly by inflating cuffs in the upper arm above venous pressure but below arterial venous pressure, hence occluding venous outflow; typical traces are shown below (Figure 2.2). Therefore, blood can flow into the forearm but it cannot drain away, thus increasing forearm volume that is proportional to arterial flow. The vascular bed in the hand is typically excluded through cuff inflation beyond systolic pressure, as it contains shunts that can confound measurements.

Figure 2.1. Participant undergoing a venous occlusion plethysmography study with a typical set up.

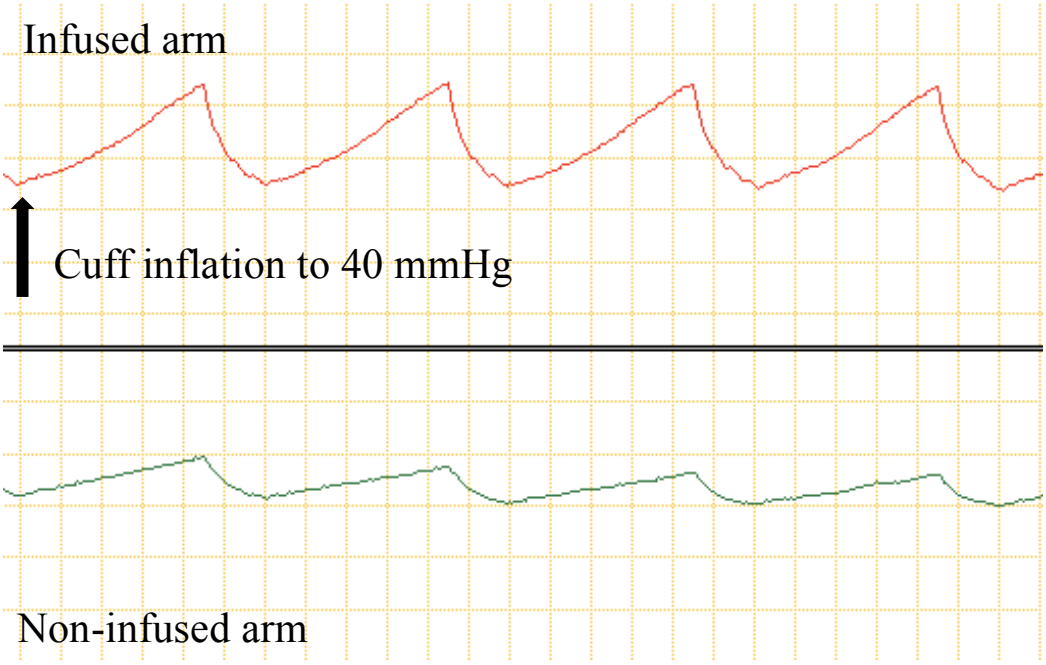

Figure 2.2. Typical traces during a plethysmography study.

2.3.2 INTRA-ARTERIAL CANNULATION

The brachial artery of the non-dominant forearm was cannulated with a 27-standard wire gauge steel needle (Coopers Needle Works Ltd, Birmingham, UK) following subcutaneous infiltration of local anaesthetic (Hameln Pharmaceuticals Ltd, Gloucester, UK). The cannula was attached to a 16-gauge epidural catheter (Portex Ltd, Hythe, UK) and patency maintained with infusion of saline (0.9%; Baxter Healthcare Ltd, Norfolk, UK) via an IVAC P6000 syringe pump (Alaris Carefusion, Basingstoke, UK) total rate of intra-arterial infusion was 1 mL/min in all studies (Figure 2.3).

2.3.3 BLOOD FLOW MEASUREMENT

Mercury-in-silastic strain gauges were applied to the widest part of the forearm. Both arms were elevated above the heart to ensure adequate venous drainage. During the assessment of blood flow, the upper arm cuffs were inflated repeatedly to 40 mmHg to occlude venous outflow for 9 out of 12 seconds. The wrist cuffs were inflated to 200 mmHg during measurement periods, in order to exclude the hand circulation. The cuffs were inflated using a rapid cuff inflation system (DE Hokanson Inc, Bellevue, WA, USA). Resistance in the strain gauge (Mercury-in-silastic; DE Hokanson Inc, USA) was measured with an EC6 strain gauge plethysmograph (DE Hokanson Inc, USA) and processed by Chart^{TM} v5.0.1 software (AD Instruments Ltd, Oxfordshire, UK).

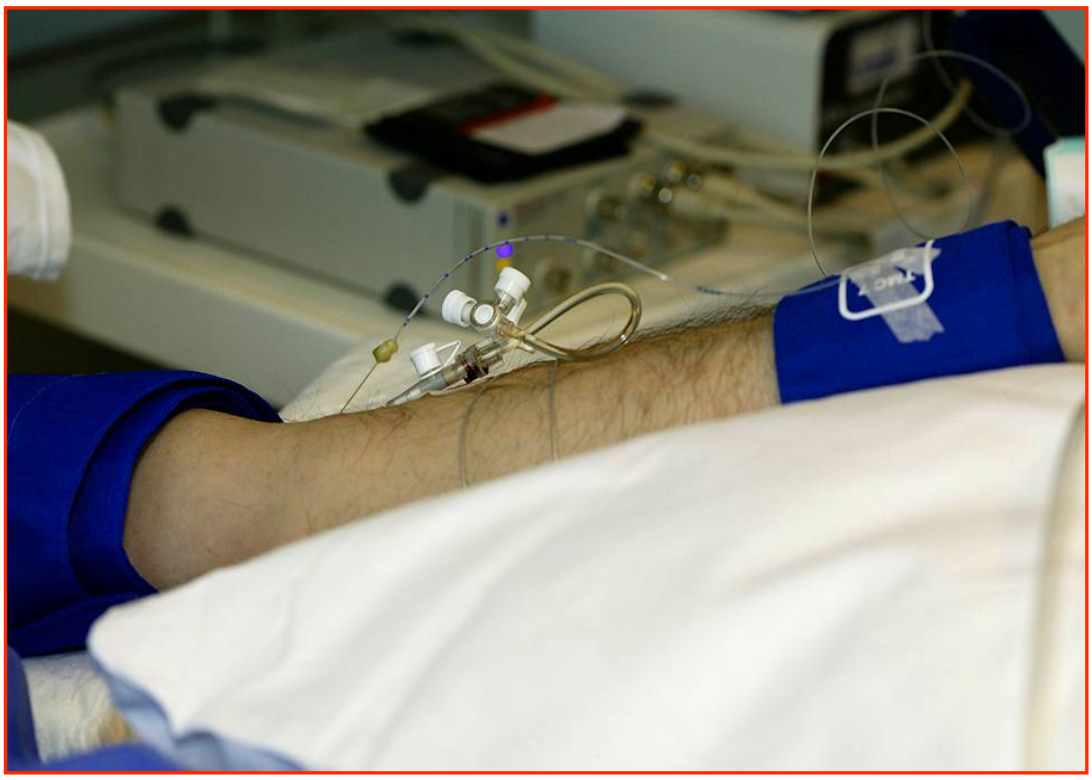

Figure 2.3. Arterial cannulation (needle) with mercury-in-silastic strain gauges placed around the forearm. Wrist cuff, which inflates to 200 mmHg to exclude hand circulation, and arm cuff, which inflates periodically to 40 mmHg to cause intermittent venous occlusion.

2.3.4 PLETHYSMOGRAPHY DATA ANALYSIS

Blood flow from each individual was analysed on Chart™ and calculated on an Excel template spread sheet (Excel 2008, Microsoft Corporation, WA, USA). Blood flow assessment was calculated from the last five recordings in each 3-minute recording period, and the average was then calculated.

2.4 ASSESSMENT OF SYSTEMIC HAEMODYNAMICS

Cardiac output is challenging to assess, and no technique is entirely satisfactory. The Fick principle is considered the gold standard in this respect. However, it is limited by the requirement for specialist face masks and arterial and venous blood samples: in the context of repeated measures and prolonged infusions of study medications, an indwelling arterial cannula would be required. Thermodilution or tracer techniques can also be used, although these too are invasive and require central venous cannulation. Thoracic electrical bioimpedance is a technique first described in 1940 that has subsequently been refined and can be used to assess cardiac output non-invasively [Nyboer *et al* 1970; Tsadok 1999].

2.4.1 THORACIC ELECTRICAL BIOIMEPEDANCE

Thoracic electrical bioimpedance is a non-invasive method for assessing cardiac output electrodes, 2 on the neck and 2 on the thorax (Figures 2.4 and 2.5), and a small current is applied to the outermost electrodes and detected on the innermost electrodes.

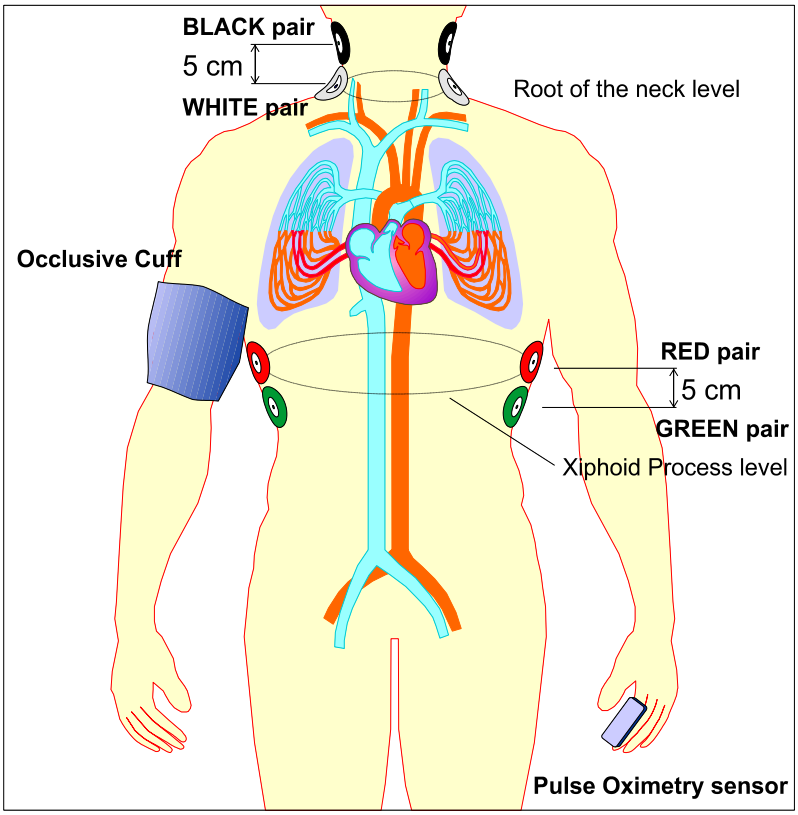

Figure 2.4. Theoretical landmarks for placement of thoracic electrical bioimpedance electrodes.

Figure 2.5. Thoracic bioimpedance electrodes on a volunteer.

Impedance, which is the opposition of charge flow in alternating current circuits, is analogous to resistance in direct current circuits. Data are acquired by applying a small current through electrodes in the neck and midaxillary line. Nyboer *et al* [1970] were the first to describe changes in thoracic impedance as inversely related to the stroke index, and this has been refined over time [Thomas 1992].

The thorax is modelled as a cylindrical conductor that has a further smaller cylinder conductor, the great vessels, within. Impedance is modelled as:

$$Z = \boldsymbol{\rho} \frac{A}{L}$$

(Z = impedance, **ρ** = specific resistivity, A = area, L = length).

Each of the component tissues within the thorax contribute to the overall resistance, the properties of which are largely unaltered throughout the cardiac cycle [Osypka and Bernstein 1999]. Of all the tissues, blood has the lowest resistance, so the current will flow through the great vessels in the thorax. Therefore, changes in impedance throughout the cardiac cycle can be considered as a function of changes of impedance within the great vessels, with almost all of the early impedance changes related to blood volume change in the aorta [Kim *et al* 1988].

Both aortic compliance and thoracic fluid content impact on impedance. Any reduction in aortic compliance, through age, for example, will reduce impedance and similarly increase fluid content in the thorax, which in turn will decrease compliance. Conditions such as emphysema, with increased air content, will increase impedance [Osypka and Bernstein 1999].

Thoracic electrical bioimpedance has been validated against accepted invasive methods of measuring cardiac output. Correlation is accepted to be high, with values ranging from 0.77 - 0.99 . [Northridge *et al* 1990; Bernstein *et al* 1986; Salandin *et al* 1988; Thomas *et al* 1992; Shoemaker *et al* 1994; Drazner *et al* 2002; Sageman 2002; Yung *et al* 2004; Engoren and Barbee 2005; Suttner *et al* 2006; Gujjar *et al* 2008; Tonelli *et al* 2011]. Mean differences between cardiac output assessed invasively and thoracic bioimpedance ranged from 2.7-9.0% [Northridge *et al* 1990; Thomas *et al* 1992]. Importantly, changes trends in cardiac output over time assessed by thoracic bioimpedance correlate to invasive techniques over time. [Shoemaker *et al* 1994] Furthermore, thoracic electrical bioimpedance can detect changes in cardiac output in response to drug infusion, which is best when considering changes from baseline rather than absolute changes [Thomas et al 1992].

There are limited data assessing thoracic electrical bioimpedance in chronic stable heart failure, although correlation with invasive techniques of assessing cardiac output exists and appears to be a suitable technique for assessing cardiac function in heart failure [Drazner *et al* 2002] and pulmonary hypertension [Tonelli *et al* 2011]. Typically, electrical bioimpedance will overestimate cardiac output in heart failure

relative to thermodilution. However, good agreement has been demonstrated with respect to assessing changes in cardiac output [Tanino *et al* 2009].

2.4.2 IMPEDANCE CARDIOGRAPHY AND SPHYGMOMANOMETRY

Cardiac index was measured non-invasively through thoracic bioimpedance (Hotman Hemo Sapiens, CA, USA). Prior to the infusion of study medication, all subjects rested in a supine position for at least 30 minutes to stabilise all measured haemodynamics, and the studies only proceeded once three recordings were within 10% of a continuous average.

At each time point, an index output was taken as the mean of three recordings, each one of which represented the average of 15 consecutive heartbeats. The cardiac index was calculated automatically by:

$$CI = \frac{CO}{BSA} \text{ (L/min/m}^2\text{)}$$

An electrocardiogram was monitored continuously, and blood pressure and heart rate were recorded with a semi-automated, non-invasive oscillometric sphygmomanometer (HEM 705CP, Omron, Tokyo, Japan). Mean arterial pressure was defined as the sum of the diastolic blood pressure and a third of the pulse pressure:

$$MAP = \frac{SBP + (DBP \times 2)}{3} \text{ mmHg}$$

Systemic vascular resistance was calculated as mean arterial pressure minus mean right atrial pressure, divided by the cardiac index:

$$\text{Systemic vascular resistance index} = \frac{80 \times (\text{mean arterial pressure} - \text{right atrial pressure})}{\text{Cardiac Index}} \text{ dynes·s/cm}^5\text{/m}^2$$

2.4.3 THORACIC ELECTRICAL BIOIMPEDANCE CARDIOGRAPHY DATA ANALYSIS

The average of three values for cardiac index, heart rate, mean arterial pressure and systemic vascular resistance were calculated on an Excel spreadsheet (Excel 2008).

2.5 ASSESSMENT OF LEFT VENTRICULAR FUNCTION

Echocardiography was performed using a Philips iE33 ultrasound scanner with 3 MHz transducer (Philips Healthcare, Best, Netherlands. Left ventricular dimensions were assessed through two-dimensional in chronic heart failure studies. All studies were performed and analysed by the British Society of Echocardiography-accredited Sonographers (SA, AW).

Fractional shortening was calculated as:

$$\text{Fractional shortening} = \frac{\text{Left ventricular end-diastolic diameter} - \text{Left ventricular end-systolic diameter}}{\text{Left ventricular end-diastolic diameter}}$$

Left ventricular ejection fraction was assessed using the Teichholz method.

2.6 CARDIOPULMONARY EXERCISE TESTING

Cardiopulmonary exercise testing is a well-established technique employed to assess cardiovascular performance. Cardiac bioimpedance is less effective in exercise as the signal is distorted through motion artefact and elevated respiratory rate. However, in addition to haemodynamic and electrocardiograph information, respiratory measures of oxygen uptake and ventilation can be captured. Sports medicine has adopted cardiopulmonary exercise testing to assess and monitor athletic performance, but in clinical medicine valuable prognostic information can be acquired from exercise testing.

In patients with heart failure, cardiopulmonary exercise testing is an excellent prognostic tool [Stelken *et al* 1996; Myers *et al* 2000; Guazzi *et al* 2005] and is widely used to stratify patients following cardiac transplantation [Mancini *et al* 1991]. Furthermore, in patients with pulmonary arterial hypertension,

cardiopulmonary exercise testing predicts survival [Wensel *et al* 2012]. Exercise testing can assess responses to therapies in healthy volunteers and patient cohorts [O'Donnell *et al* 2004; Lewis *et al* 2006; de Man *et al* 2009; Li *et al* 2009; Vagaggini *et al* 2011]. Typically, an incremental exercise test is performed to determine the maximal workload of an individual, followed by endurance tests that are performed at 70-80% of maximal workload, with and without intervention.

The main cardiopulmonary indices are reproducible on repeated testing. In healthy, trained individuals, low intra-subject variability is reported in sequential testing [Weston and Gabbett 2001] and these findings extend to patient populations with heart failure and pulmonary disease [Meyer *et al* 1997; Hansen *et al* 2004; Keteyian *et al* 2010]. This is not universal, though, and some investigators report high intra-subject variations in repeated testing. Furthermore, on repeated testing, some studies report the equal likelihood of improvement or deterioration, suggesting that learning and conditioning are not major determinants of performance [Bensimhon *et al* 2008].

Cardiopulmonary exercise tests were performed on a cycle ergometer (ViaSYS Healthcare, Carefusion, Basingstoke, UK) at Imperial College NHS Healthcare Trust's exercise laboratory. Ventilation and gas exchange parameters were measured continually producing a breath-by-breath analysis of carbon dioxide and oxygen during exercise protocols. Breath-by-breath analyses were recorded throughout (Master Screen CPX Metabolic Cart, Carefusion, Basingstoke, UK) and data were collected automatically (JLAB LABManager, software version 5.3.0.4, Cardinal Health Germany, Hoechberg, Germany), Baseline pulmonary function was assessed

prior to exercise testing. Blood pressure was assessed at regular intervals during exercise and there was continuous electrocardiograph monitoring throughout each visit. Oxygen consumption rate (VO_2), carbon dioxide production (VCO_2), tidal volume (V_T) and ventilation were measured throughout all exercise protocols.

2.6.1 INCREMENTAL MAXIMAL CARDIOPULMONARY EXERCISE PROTOCOL

Maximal workload was determined by incremental exercise testing. An initial rest period with the subjects seated on an ergometer was followed by 1 minute of unload cycling and thereafter an incremental increase in workload of 30 watts per minute, at 1 watt increments, was applied until symptom limitation was reached. Patients were encouraged to maintain maximal workload, and maximum effort was determined by any of the following: 85% of maximum predicted heart rate, respiratory exchange ratio >1.1 or a VO_{2MAX} plateau accepted as maximal effort.

2.6.2 ENDURANCE CARDIOPULMONARY EXERCISE PROTOCOL

Following incremental exercise tests, subjects attended on two further occasions to undertake an endurance exercise protocol. This was set at 80% of maximal work rate, as determined from the initial incremental exercise test. Following 2 minutes of rest, the subjects exercised with no resistance for 1 minute, and thereafter the appropriate workload was applied rapidly.

2.7 VENOUS SAMPLING AND LABORATORIES

2.7.1 FOREARM VENOUS SAMPLING

Venous cannulae (17-gauge) were inserted into the large antecubital veins of both arms to allow for drug infusion and the sampling of venous blood. Blood samples were drawn into ethylene diamine tetraacetic acid (EDTA) or serum gel (Monovette®, Sarstedt, Nümbrecht, Germany).

2.7.2 URINE COLLECTION

Urine was collected in non-sterile acidified containers and analysed for sodium content (Department of Clinical Biochemistry, Lothian NHS University Hospitals Trust, Scotland).

2.7.3 SAMPLE PREPARATION

Blood samples were drawn into EDTA, centrifuged at 2000 *g* for 15 minutes to obtain plasma and stored at -80^{o}C until assayed. Subjects voided prior to commencing all studies and urine was collected throughout the systemic studies. Urinary sodium concentration was determined using an ion selective electrode.

2.7.4 ANGIOTENSIN II AND PLASMA RENIN ACTIVITY ASSAYS

Plasma angiotensin II concentrations (Peninsula Laboratories Europe Ltd, St Helens, UK) were determined by radioimmunoassay, following extraction using Bond Elut® columns (Agilent Technologies, Cheshire, UK). Plasma renin activity was measured under standard conditions through the generation of angiotensin I, as determined by radioimmunoassay. Urinary sodium concentration was determined using an ion selective electrode.

2.7.5 PLASMA APELIN CONCENTRATION

Plasma apelin was assayed using standard, commercially available enzyme-linked immunosorbent assay (ELISA) (Phoenix Peptides, CA, USA) and processed in accordance with the manufacturer's instructions.

2.8 DATA ANALYSIS

2.8.1 IMPEDANCE CARDIOGRAPHY AND SPHYGMOMANOMETRY ANALYSIS

An average of three values for cardiac index, heart rate, mean arterial pressure and systemic vascular resistance was calculated on an Excel spreadsheet (Excel 2008). Data were analysed by analysis of variance (ANOVA) and paired two-tailed Student's *t*-test using GraphPad Prism (GraphPad Software Inc, CA, USA) and presented as the mean and standard error of the mean (SEM), unless otherwise stated. Statistical significance was taken at the level of 5%.

2.8.2 PLETHYSMOGRAPHY DATA ANALYSIS

Blood flow from each individual was analysed on Chart^{TM} and calculated on an Excel template spread sheet (Excel 2008). Blood flow assessment was calculated from the last five recordings in each 3-minute recording period, and the average was then calculated. Data were analysed by ANOVA and paired two-tailed Student's *t*-test using GraphPad Prism and presented as the mean and SEM, unless otherwise stated. Statistical significance was taken at the level of 5%.

2.8.3 CARDIOPULMONARY EXERCISE TEST

Absolute values of VO_{2MAX}, mean arterial pressure, ventilation efficiency, endurance time and heart rate during (Pyr[1])apelin-13 infusion will be compared with those during the placebo infusion by paired two-tailed Student's *t*-test. All data were analysed using GraphPad Prism and presented as the mean and SEM, unless otherwise stated. Statistical significance was taken at the level of 5%.

2.8.4 ASSAYS

Data were analysed by paired two-tailed Student's *t*-test using GraphPad Prism and presented as the mean and SEM, unless otherwise stated. Statistical significance was taken at the level of 5%.

2.8.5 DRUGS

APLNR agonism was investigated with pharmaceutical grade (Pyr[1])apelin-13 (Clinalfa AG, Läufelfingen, Switzerland, or Genscript, NJ, USA). Dose ranges were ascertained from previous studies performed within the group that produced maximal physiological effects in techniques used. Previously forearm studies assessing local arterial responses to (Pyr[1])apelin-13 were preformed over a three hundred fold range in concentration, from 0.1nanmol/min to 30nanomaol/min. No further local vasodilatation was observed beyond 3nanomol/min and dosing in acute local studies was derived from these data [Japp *et al* 2010]. Systemic infusions protocol doses were based on previous studies within the group and from my own experience during

studies. Systemic infusion dose response curves in healthy volunteers and patients with chronic stable heart failure demonstrated no efficacy beyond 30nanmol/min and therefore this dose was selected for prolonged infusion. [Japp *et al* 2011].

Two different apelin peptide suppliers were used during the studies presented in this thesis, Genscript and Bachem. Genscript peptide was used in all studies performed in University of Edinburgh, (protocols in chapter 3-6) and Bachem peptide was used in studies performed in Imperial college London. Potency of Genscript peptide was assessed during one unblended local arterial infusion study of (Pyr^{1})apelin-13 that yielded similar results to previous local studies performed within the group. Furthermore, on completion for the first protocol, results were reviewed internally and, again, vascular response to Genscript peptide were similar to prior studies within the group. Bachem peptide was not re-evaluated and assumed to be of similar potency to previous studies.

Protocols requiring angiotensin II infusions were conducted with pharmaceutical grade angiotensin II (Clinalfa AG, Läufelfingen, Switzerland). Control vasodilators were acetylcholine (Norvartis AG, Basel, Switzerland), sodium nitroprusside (Mayne Pharma Plc, Warwickshire, UK).

CHAPTER 3

LOCAL AND SYSTEMIC VASCULAR RESPONSES TO APELIN RECEPTOR AGONISM DURING SUBACUTE RENIN-ANGIOTENSIN ACTIVATION

Barnes GD, Alam S, Carter G *et al.*
Sustained cardiovascular actions of APJ agonism during renin-angiotensin system activation and in patients with heart failure.
Circ Heart Fail 2013;**6**(3):482-491.

3.1 SUMMARY

Introduction Expression of the Apelin-APLNR system is reduced in conditions of renin-angiotensin system activation. This study assessed local and systemic cardiovascular actions of (Pyr^{1})apelin-13 during subacute renin-angiotensin elevation.

Methods In a single blinded randomised crossover trial, patients were allocated a low sodium diet (<12 mmol sodium/day) or a normal diet, with visits separated by at least one week. Forearm blood flow was assessed with venous occlusion plethysmography during intra-arterial (Pyr^{1})apelin-13 (0.3, 1.0 and 3.0 nmol/min), acetylcholine (5, 10 and 20 μg/min) and sodium nitroprusside (0.5, 1.0 and 2.0 μg/min) infusion. Cardiac index, systemic vascular resistance index, heart rate and mean arterial pressure were assessed with thoracic electrical bioimpedance and a semi-automated sphygmomanometer respectively during systemic (Pyr^{1})apelin-13 (30, 100 and 300 nmol/min) or placebo infusion (0.9% saline). Plasma renin activity and angiotensin concentrations were measured at the beginning of each visit.

Results Sodium depletion increases plasma renin activity (4.2±0.9 *versus* 0.8±0.2 ng/mL/hr; P <0.01) and plasma angiotensin II concentrations (11.6±1.9 *versus* 5.1±1.0 pg/mL; P <0.01). All local and systemic vascular responses to (Pyr^{1})apelin-13 infusion were unaffected by systemic angiotensin II plasma concentration.

Conclusion During significant upregulation of the renin-angiotensin system cardiovascular responses to (Pyr^1)apelin-13 are preserved. The apelin-APLNR system is predicted to replicate these results in disease states with increased renin-angiotensin activation notably heart failure.

3.2 INTRODUCTION

The G protein-coupled receptor, APLNR, was identified in 1993 (O'Dowd *et al* 1993), and of all G protein-coupled receptors it most closely resembles the angiotensin II type 1 receptor. These receptors share around a 50% sequence homology in the transmembrane domains and are present in similar tissue locations throughout the body [Lee *et al* 2000; Ashley *et al* 2006]. However, these two systems mediate opposing actions with regard to inflammation [Chun *et al* 2008], vascular tone [Gurzu *et al* 2006] and fluid balance [Azizi *et al* 2008].

The apelin-APLNR system is vasoactive and mediates vasodilatation [Lee *et al* 2000] that is endothelial and nitric oxide-dependent [Tatemoto *et al* 1998; Hashimoto *et al* 2006; Jia *et al* 2007; Zhong *et al* 2007b). In cultured aortic tissue, apelin increases nitric oxide production in a dose-dependent manner, increasing nitric oxide activity and expression whilst promoting *L*-arginine transport [Jia *et al* 2007]. Apelin mediates vasorelaxation in myography studies [Salcedo *et al* 2007], although vasoconstriction is also reported in denuded human saphenous veins [Katugampola *et al* 2001]. In whole animal models, the administration of apelin results in rapid transient reduction in mean arterial pressure

[Lee *et al* 2000] and increases plasma nitrate and nitrite levels [Tatemoto *et al* 2001]. In man, local regional apelin infusion in the brachial arterial and coronary arterial circulation produces vasodilatation, whilst systemic infusions reduce mean arterial pressure [Japp *et al* 2008; Japp *et al* 2010].

The inotropic action of apelin has been characterised in a range of preclinical models. In isolated cardiomyocytes it is reported to be the most potent inotrope discovered, effective at subnanomolar concentrations [Szokodi *et al* 2002]. Furthermore, the actions of apelin on failing myocardium appear to be more potent than normal myocardium [Dai *et al* 2006; Farkasfalvi *et al* 2007]. In whole perfused hearts and animal models, apelin increases cardiac output, and importantly under constant loading conditions there is increased contractility; therefore, inotropic action is in part through direct myocardial action rather than reducing afterload [Ashley *et al* 2005]. Whilst clinical studies are limited, data from *in vivo* studies confirm its inotropic action in man [Japp *et al* 2010].

The interaction between apelin and renin-angiotensin is essential to understand, with evidence from preclinical studies suggesting that there is significant interaction between these two systems. Beyond the opposing actions in vascular tone, fluid homeostasis and inflammation, there appear to be important subcellular interactions [Chun *et al* 2008; Sun *et al* 2011]. The formation of APLNR and AT1R heterodimers has been reported in preclinical models, with important functional consequences. Critically, the non-activated APLNR is posited to mediate angiotensin II antagonism, with the activated APLNR potentiating AT1R signalling [Sun *et al* 2011].

Furthermore, in a range of preclinical studies, angiotensin II elevations downregulate apelin-APLNR system expression [Ishida *et al* 2004; Iwanaga *et al* 2006]. Therefore, these two systems interact at physiological, receptor and subcellular levels. The aim of this study is to assess the effect of subacute renin-angiotensin elevation on the local and systemic vascular actions of APLNR agonism.

3.2.1 HYPOTHESIS

Subacute upregulation of the renin-angiotensin system will reduce the cardiovascular effects of APLNR agonism in local peripheral and systemic circulation.

3.3 METHODS

3.3.1 SUBJECTS

Twelve healthy volunteers, aged between 19 and 22 years, participated in these studies, all of which were performed with the approval of the local Ethics Research Committee, in accordance with the Declaration of Helsinki and with the written consent of all volunteers. Subjects were excluded if they were receiving any regular medication, had any significant past medical history, were current smokers or had participated in research studies within 3 months of enrolment.

3.3.2 STUDY DESIGN

Twelve subjects attended on two occasions, at least one week apart after being randomised in a single blind crossover design to a sodium deplete or normal sodium replete diet, as described previously (Figure 3.1) [Newby *et al* 1997b].

Briefly, for sodium depletion, subjects were asked to adhere to a diet containing <12 mmol of sodium per day for 3 days prior to the study visit (Appendix, p 186). To ensure prompt sodium depletion, subjects were given a single oral dose of furosemide (20 mg) on day one of the diet. For all sodium depletion studies, urine was collected from each individual 24 hours before each visit, in order to assess sodium excretion. Plasma renin activity and plasma angiotensin II concentrations were assessed following dietary modifications prior to the beginning of each study visit. At each visit subjects had local and systemic cardiovascular studies performed. All subjects abstained from alcohol and caffeine for 24 hours and food from 4 hours prior to their study visit. All studies were performed in a quiet, temperature controlled room maintained at 22-24°C with subjects lying supine. There was minimal mobilisation between the local and systemic vascular studies.

***Protocol 1*:**

All subjects underwent brachial artery cannulation with a 27-standard wire gauge steel needle under aseptic conditions. After a 30-minute baseline 0.9% saline infusion, forearm blood flow was assessed following intra-arterial infusion of (Pyr^{1})apelin-13 (0.3, 1.0 and 3.0 nmol/min), acetylcholine (5, 10 and 20 μg/min) and sodium nitroprusside (0.5, 1.0 and 2.0 μg/min) given in a double blind randomised manner, [Japp *et al* 2008] with each infusion given for 6 minutes (Figure 3.1). Dosages of intra-arterial (Pyr^{1})apelin-13 were determined from previous studies within our group [Japp *et al* 2008; Japp *et al* 2010]. The sequence of vasodilator administration was randomised between subjects but was kept constant for each

individual subject, in order to maintain a consistent order for both study visits, with 30-minute saline infusions separating each vasodilator.

Forearm blood flow was measured in the infused and non-infused arms by venous occlusion plethysmography, as described previously [Newby *et al* 1997a). Supine heart rates and blood pressure were measured in the non-infused arm at regular intervals during the study.

***Protocol 2*:**

Cardiac index, peripheral vascular resistance, mean arterial pressure and heart rate were measured every 5 minutes during a 0.9% saline infusion using thoracic impedance cardiography and a semi-automated non-invasive sphygmomanometer, as described previously [Thomas 1992]. Once all readings were within 10% of a rolling average and only after a minimum of a 30-minute run-in infusion, cardiac index, peripheral vascular resistance, mean arterial pressure and heart rate were measured during systemic (Pyr^{1})apelin-13 infusion (30, 100 and 300 nmol/min) or placebo, administered for 6 minutes (Figure 3.2). (Pyr^{1})apelin-13 and placebo infusion were separated by a 30-minute washout period. Infusions were administered in a randomised double blinded manner. The dose of (Pyr^{1})apelin-13 was determined from previous studies within our group [Japp *et al* 2010].

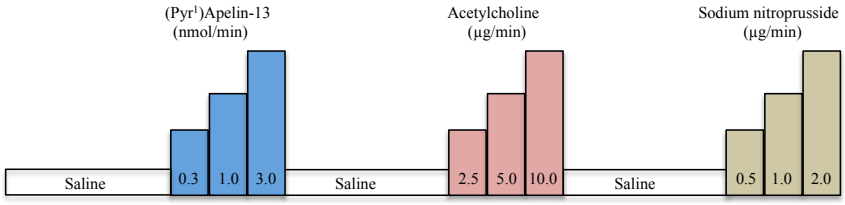

Figure 3.1. Study design for the local vascular studies.

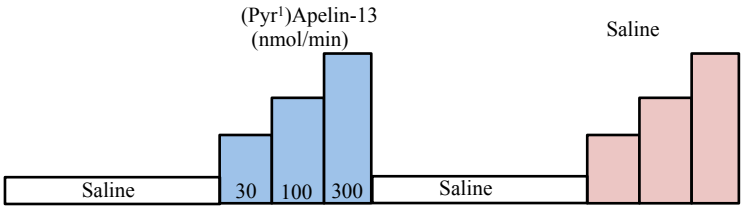

Figure 3.2. Study protocol for the systemic haemodynamic studies.

3.3.3 DATA AND STATISTICAL ANALYSIS

Variables are reported as mean±SEM and analysed using repeated measures ANOVA with post-hoc Bonferroni corrections and paired two-tailed Student's *t*-test as appropriate (GraphPad Prism). Forearm blood flow was calculated from plethysmographic data, as described previously [Newby *et al* 1998; Japp *et al* 2008]. Mean arterial pressure was defined as the sum of the diastolic blood pressure and a third of the pulse pressure. Peripheral vascular resistance index was calculated as mean arterial pressure minus mean right atrial pressure, divided by cardiac index. Statistical significance was taken as $P < 0.05$. Based on power calculations derived from previous studies [Japp *et al* 2010] and a significance level of 5%, the sample sizes (n=12) will give 90% power of detecting the clinically meaningful differences of 0.7 mL/100 mL per minute and 0.6 L/min in forearm blood flow and cardiac output, respectively. We have previously described the influence of a range of factors on regional and systemic vascular beds using sample sizes of 8 to 12 subjects. (Japp *et al* 2008, Japp *et al* 2010 , Newby *et al* 1996].

3.4 RESULTS

3.4.1 STUDY PARTICIPANTS

Healthy volunteers were male, aged 21±0 years with a body mass index of 22±1 kg/m^2. All studies were well tolerated and progressed without any serious adverse events.

3.4.2 RENIN-ANGIOTENSIN SYSTEM ACTIVATION: SODIUM DEPLETION

Subjects adhered to the sodium depleted diet (Appendix, p 186), as demonstrated by a marked reduction in urinary sodium excretion (34±6 *versus* 175±21 mmol/day; P <0.0001) and increases in both plasma renin activity (4.2±0.9 *versus* 0.8±0.2 ng/mL/hr; P <0.01) and plasma angiotensin II concentrations (11.6±1.9 *versus* 5.1±1.0 pg/mL; P <0.01) in comparison to the sodium replete diet (Figure 3.3).

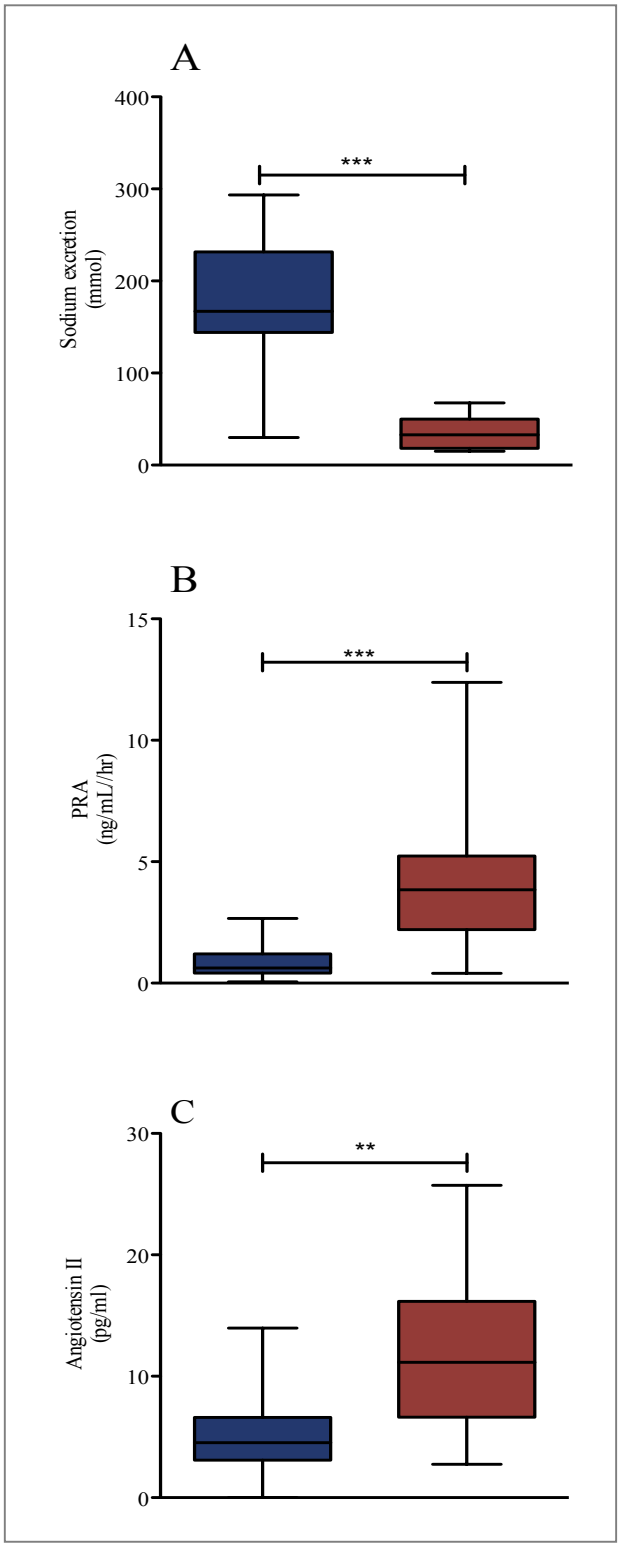

Figure 3.3. A) Total 24-hour urinary sodium excretion (mmol) during sodium replete diet (blue) or sodium depleted diet. **B)** Plasma renin activity (PRA) (ng/mL/hr) during sodium replete diet (blue) or sodium deplete diet (red). **C)** Plasma angiotensin II concentrations (pg/mL) during sodium replete diet (blue) or sodium deplete diet (red). Paired Student's *t*-test (**P* <0.5, ***P* <0.01, ****P* <0.0001).

3.4.3 PERIPHERAL RESISTANCE VESSELS

Local APLNR agonism was assessed with (Pyr[1])apelin-13 (Genscript). There were no changes in heart rate, blood pressure or non-infused forearm blood flow throughout all the studies.

Baseline forearm blood flow was unchanged by sodium depletion (1.9±0.2 *versus* 1.9±0.2 mL/100 mL/min; $P > 0.05$). Both (Pyr[1])apelin-13 and sodium nitroprusside caused vasodilatation in the infused arm ($P < 0.001$ for all), and these responses were similar under both dietary conditions ($P > 0.05$ for sodium deplete *versus* sodium replete diet; Figure 3.4). Acetylcholine caused dose-dependent vasodilatation under both dietary conditions ($P < 0.001$ for all), and was attenuated, but not abolished, during sodium depletion ($P < 0.001$, sodium deplete *versus* sodium replete diet).

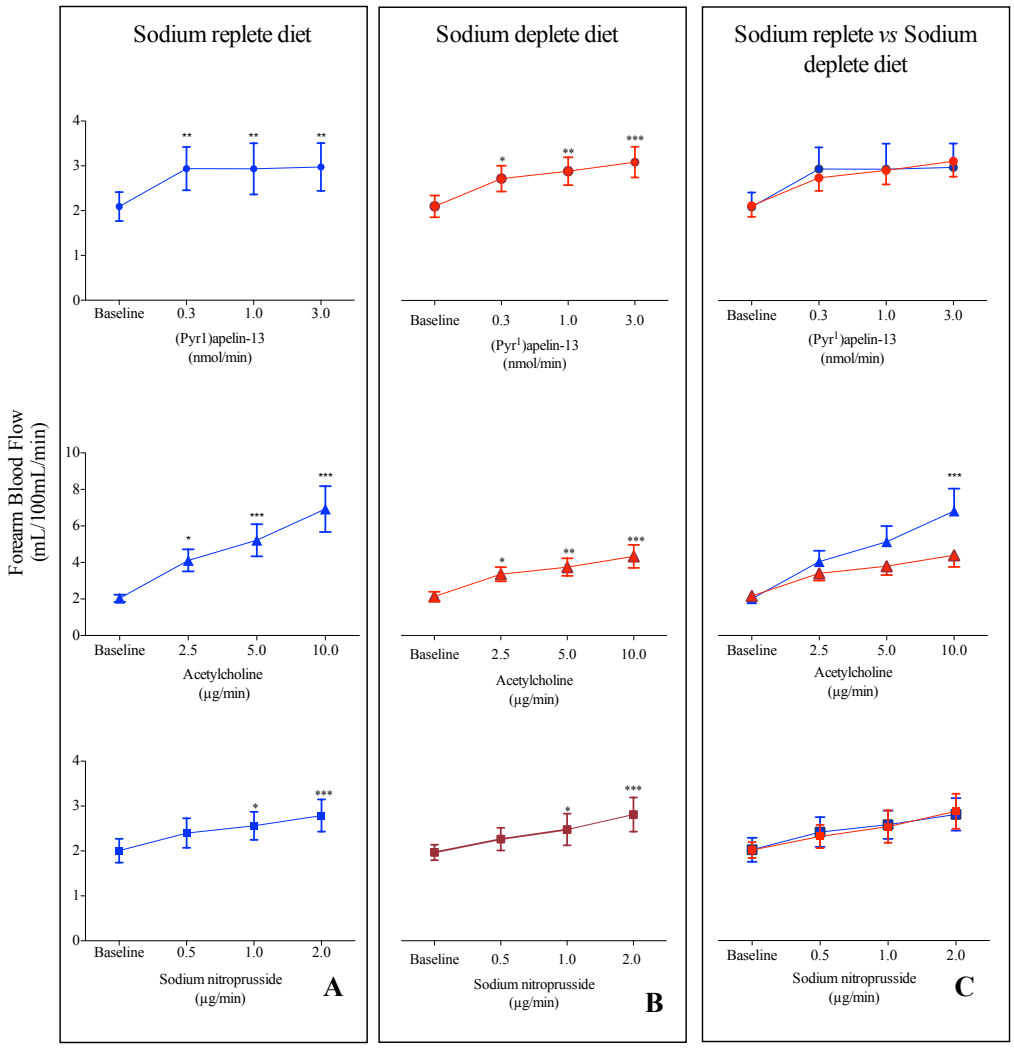

Figure 3.4. A) Sodium replete diet: forearm blood flow (mL/100mL/min) during intra-arterial (Pyr[1])apelin-13 (blue circles, top), acetylcholine (blue triangles, middle) or sodium nitroprusside (blue squares, bottom). **P* <0.05, ***P* <0.01, ****P* <0.001. Two-way ANOVA with post-hoc Bonferroni test. **B) Sodium deplete diet:** forearm blood flow (mL/100mL/min) during intra-arterial (Pyr[1])apelin-13 (red circles, top), acetylcholine (red triangles, middle) or sodium nitroprusside (red squares, bottom). *P* <0.05, ***P* <0.01, ****P* <0.001. Two-way ANOVA with post-hoc Bonferroni test. **C)** Comparison of sodium replete (blue, all) and sodium deplete (red, all) diets. Forearm blood flow (mL/100mL/min) during intra-arterial (Pyr[1])apelin-13 (circles, top), acetylcholine (triangles, middle) or sodium nitroprusside (squares, bottom). **P* <0.05, ***P* <0.01, ****P* <0.001. Two-way ANOVA with post-hoc Bonferroni test. ANOVA - analysis of variance.

3.4.4 SYSTEMIC APLNR AGONISM

Systemic APLNR agonism was assess with (Pyr^{1})apelin-13 (Genscript). Baseline haemodynamic data are presented in Table 3.1, with no differences observed at baseline. Intravenous (Pyr^{1})apelin-13 infusion increased cardiac index (P <0.001 for both), reduced mean arterial pressure (P <0.02 for both) and reduced peripheral vascular resistance index (P <0.001 for both) compared to matched placebo with no differences between the sodium deplete and sodium replete diets (Figure 3.5). A trend to increased heart rate was evident following the sodium replete diet (P=0.06) that was not present during sodium depletion. In keeping with our previous work [Japp *et al* 2010], (Pyr^{1})apelin-13 rapidly achieved its peak systemic haemodynamic effects, with a plateau in the dose response curve evident.

TABLE 3.1 Baseline haemodynamic data

	Sodium replete diet	Sodium deplete diet
Cardiac Index L/min/m^2	4.0±0.2	4.1±0.3
Heart Rate beats/min	60.8±2.6	62.3±4.0
Systolic Blood Pressure mmHg	128.4±4.9	124.8±3.1
Diastolic Blood Pressure mmHg	72.3±2.7	70.0±1.4
Mean Arterial Pressure mmHg	89.9±2.9	88.2±1.6
Peripheral Vascular Resistance Index dynes.s/cm^5/m^2	1737±83	1728±114

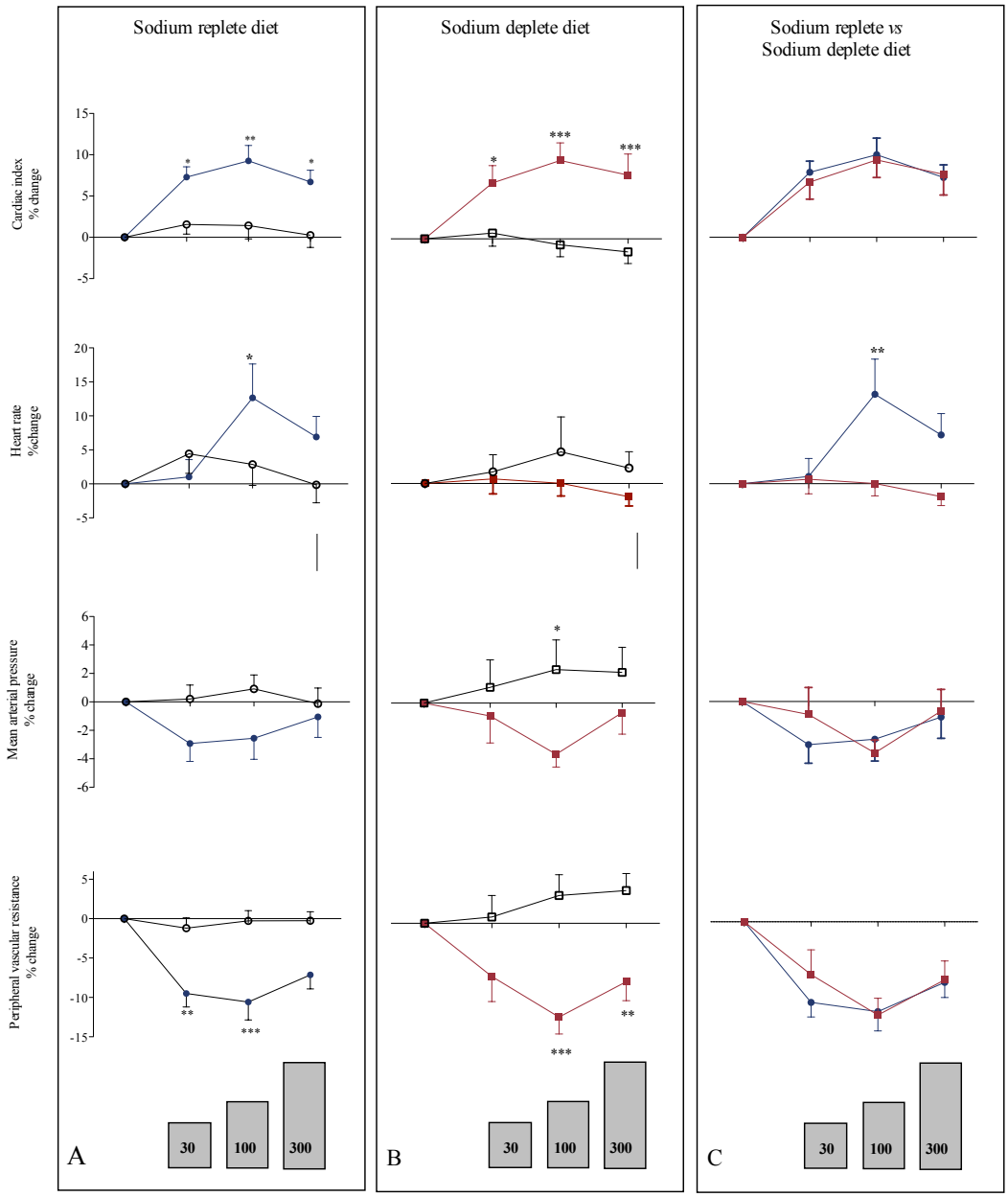

Figure 3.5. A) Sodium replete diet: Percentage change from baseline in cardiac index, heart rate, mean arterial pressure and peripheral vascular resistance during intravenous (Pyr[1])apelin-13 (blue circles, closed) or placebo (black circles, open. **P* <0.05, ***P* <0.01, ****P* <0.001. Two-way ANOVA with post-hoc Bonferroni test. **B) Sodium deplete diet:** Percentage change from baseline in cardiac index, heart rate, mean arterial pressure and peripheral vascular resistance during intravenous (Pyr[1])apelin-13 (red squares, closed) or placebo (black squares, open). **P* <0.05, ***P* <0.01, ****P* <0.001. Two-way ANOVA with post-hoc Bonferroni test. **C) Comparison of sodium replete (blue, all) and sodium deplete (red, all) diets:** Percentage change from baseline in cardiac index, heart rate, mean arterial pressure and peripheral vascular resistance during intravenous (Pyr[1])apelin-13 (blue squares closed, sodium replete diet; red squares, closed, sodium deplete) . **P* <0.05, ***P* <0.01, ****P* <0.001. Two-way ANOVA with post-hoc Bonferroni test.
ANOVA-analysis of variance.

3.5 DISCUSSION

This is the first study in man to assess the effect of APLNR agonism under conditions of subacute renin-angiotensin activation. During these studies the local and systemic effects of APLNR agonism were preserved and, as such, the effect of (Pyr^{1})apelin-13 are predicted to retain their efficacy under conditions of renin-angiotensin system upregulation. No physiologically relevant interaction between these two hormone systems was identified in these studies.

In this study there was good adherence to the sodium depleted diet, as reflected by changes in sodium excretion (34±6 *versus* 175±21 mmol/day; P <0.0001), with corresponding upregulation of the renin-angiotensin system demonstrated by increased plasma renin activity (4.2±0.9 *versus* 0.8±0.2 ng/mL/hr; P <0.01) and plasma angiotensin II concentrations (11.6±1.9 *versus* 5.1±1.0 pg/mL; P <0.01) compared to the sodium replete diet. Therefore, the lack of interaction, we feel, does not reflect insufficient upregulation of the renin-angiotensin system. Furthermore, the timescale and extent of renin-angiotensin system activation are sufficient to alter apelin-APLNR expression. Preclinical data suggest that angiotensin II results in apelin downregulation within 24 hours, even at subpressor doses [Iwanaga *et al* 2006].

In contrast to (Pyr^{1})apelin-13, acetylcholine-induced vasodilatation was impaired following sodium depletion and renin-angiotensin system activation, suggesting the

presence of endothelial dysfunction. *In vitro* angiotensin II promotes superoxide anion generation when incubated with vascular smooth muscle cells [Griendling *et al* 1994]. Furthermore, in whole animals treated with angiotensin II, superoxide generation is increased and vasorelaxation to acetylcholine is impaired [Rajagopalan *et al* 1996]. The effect of dietary sodium on endothelial function has previously been assessed in both healthy individuals and patient populations [Stein *et al* 1995; Miyoshi *et al* 1997; Higashi *et al* 2001; Omland *et al* 2001; Tzemos *et al* 2008], which has led to diverse findings, with some investigators reporting the induction of vascular dysfunction with sodium loading [Miyoshi *et al* 1997] and no effect of sodium depletion [Higashi *et al* 2001; Omland *et al* 2001]. This appears to reflect the incorporation of differing patient groups, such as those with hypertension [Higashi *et al* 2001], with prolonged renin-angiotensin activation and alterations in vascular function dominated by factors other than those seen in short term dietary manipulation.

There is growing evidence of an interaction between the apelin-APLNR and renin-angiotensin systems that extends beyond their opposing physiological actions. The transcription of apelin is reduced under conditions of angiotensin II elevation [Iwanaga *et al* 2006], which is evident within 24 hours and at subpressor doses. In preclinical models of vascular smooth muscle, transcription of the AT1R can be inhibited by ATRA. When hypertensive rats, which have elevated renin-angiotensin system activity, are treated with ATRA there is an elevation in APLNR expression, an increase in plasma nitrates and a fall in blood pressure, accompanied by a reduction in AT1R expression [Zhong *et al* 2005]. Whilst not assessed in a single

study, mechanical stretch reduces APLNR expression within 24 hours [Szokodi *et al* 2002] and other groups report an increase in the AT1R in similar models over a similar time frame [Kijima *et al* 1996]. These data suggest that the transcriptions of the AT1R and APLNR expression are closely related.

In knockout animal studies, the interaction of the apelin-APLNR system has been investigated, and it appears that the APLNR modifies vascular responses to angiotensin II; in APLNR-deficient animal models there is an exaggerated pressor response to angiotensin II, whilst double AT1R and APLNR knockout animals have elevated blood pressure relative to AT1R-deficient mice [Ishida *et al* 2004]. However, we have demonstrated that the haemodynamic cardiovascular effects of apelin are preserved in the presence of subacute renin-angiotensin system activation. This suggests that there does not appear to be a major functional interaction *in vivo* in man although we cannot exclude such an interaction in other settings.

3.5.1 LIMITATIONS

No evidence of an interaction between these two systems has been identified in the sub-acute setting. Whilst we do not believe the study was underpowered, it is possible that an interaction is present but below the limits of detection by the techniques used; alternatively, it may be sufficiently small that more individuals would need to be studied.

The extent of renin-angiotensin II activation was assessed on the third day through plasma renin-activity, plasma angiotensin II concentrations and urinary sodium

excretion, and we believe that on the day of study we achieved sufficient renin-angiotensin activation. We assumed that upregulation in renin-angiotensin activity would impact sufficiently on apelin-APLNR activity and expression, and crucially this serves to model diseases that are characterised by renin-angiotensin activity, such as heart failure. What is less clear from these studies is the tissue expression and activity of the APLNR and AT1R; there would have been no easy method available to quantify this. Preclinical evidence supports the role of increased AT1R expression in response to elevated angiotensin II, and this may have been relevant [Cheng *et al* 1995].

There is a finite time period that permits adherence to the strict dietary protocol required for renin-angiotensin system activation. The aim of this particular protocol was to assess the interaction between the renin-angiotensin system and the apelin-APLNR system over a period that we believed would be sufficient to modify apelin-APLNR system downregulation, and we believe that this protocol addressed that hypothesis. It is possible that these two systems may interact over a longer duration, and evidently this possibility has not been addressed by the present study.

Additionally, changes in the renin-angiotensin axis and alterations in dietary sodium may have impacted upon other hormone systems, notably vasopressin release. Both preclinical and clinical studies have highlighted that apelin release is related to vasopressin release [Azizi *et al* 2008], and there appears to be a reciprocal relationship between these two hormone systems. However, it is impossible *in vivo* in humans to alter one hormone system in isolation. Furthermore, upregulating the

renin-angiotensin system, as we did in this protocol, may more accurately reflect heart failure, which exhibits an over activation of several hormone systems [Rouleau *et al* 1991; Rouleau *et al* 1993; Kirlin *et al* 1995].

In summary, the cardiovascular effects of (Pyr^{1})apelin-13 are preserved during subacute renin-angiotensin activation and there does not appear to be a significant physiological interaction between these two systems over the time period investigated. From these studies, we predict that the apelin-APLNR system will retain efficacy in chronic stable heart failure, while the APLNR is an attractive therapeutic target for cardiovascular disease.

CHAPTER 4

LOCAL AND SYSTEMIC VASCULAR RESPONSES TO APELIN RECEPTOR AGONISM DURING ACUTE ANGIOTENSIN II INFUSION

4.1 SUMMARY

Introduction In preclinical models (Pyr[1])apelin-13 reverses angiotensin II vasoconstriction. However, some studies report increased angiotensin II signalling in the presence of APLNR activation. The aim of this study was to assess the local and systemic vascular response to (Pyr[1])apelin-13 infusion during acute angiotensin II infusion.

Methods Forearm blood flow was assessed with venous occlusion plethysmography during intra-arterial (Pyr[1])apelin-13 (0.3, 1.0 and 3.0 nmol/min), or sodium nitroprusside (2.0, 4.0 and 8.0 μmol/min), administered in a randomised double blinded design, during background angiotensin II infusion, which was titrated to reduce baseline forearm blood flow by ~50%. Thereafter, on different study visits separated by at least one week, participants were randomised in a crossover double blinded design to systemic subpressor angiotensin II (0.5 μg/kg/min) or a placebo (0.9% saline), with cardiac index, systemic vascular resistance index, heart rate and mean arterial pressure assessed by thoracic electrical bioimpedance and a semi-automated sphygmomanometer respectively during systemic (Pyr[1])apelin-13 infusion (30, 100 and 300 nmol/min) and placebo (0.9% saline) administered in a double blinded randomised design.

Results Local vasodilator effect of intra-arterial (Pyr[1])apelin-13 was retained following angiotensin II vasoconstriction. Similarly, the systemic response to

intravenous (Pyr^{1})apelin-13 infusion was unaffected by co-administration of the systemic angiotensin II infusion.

Conclusion Local and systemic actions of (Pyr^{1})apelin-13 are unaffected by acute angiotensin II infusion. There are no data from this study to support the hypothesis that APLNR agonism potentiates AT1R response.

4.2 INTRODUCTION

The apelin receptor is a G protein-coupled receptor which most closely resembles the AT1R, as they share around a 50% sequence homology in the transmembrane domains and have a similar tissue location [O'Dowd *et al* 1993]. However, angiotensin II does not bind to the APLNR, and it appears that these two receptors mediate opposing actions on fluid balance [Azizi *et al* 2008], inflammation [Chun *et al* 2008] and vascular tone [Gurzu *et al* 2006; Zhong *et al* 2007a].

There is increasing evidence that the APLNR and AT1R have important interactions. The angiotensin II type 1 receptor has been shown to form heterodimers with the bradykinin type 1 receptor [AbdAlla *et al* 2000; AbdAlla *et al* 2001; AbdAlla *et al* 2005], and the formation of these complexes modifies monoreceptor responses. When the AT1R is co-expressed with the bradykinin receptor, the efficacy of angiotensin II signalling is increased [AbdAlla *et al* 2000]. Conversely, the expression of the angiotensin II type 2 receptor results in heterodimer formation with the AT1R, an interaction that inhibits angiotensin II response [AbdAlla *et al* 2001].

These data suggest that there is vital communication between G protein-coupled receptors that influences cellular signalling. Given the structural and functional properties of apelin-APLNR and the renin-angiotensin system, interactions between APLNR and the AT1R have been investigated. Preclinical studies support that the formation of AT1R and APLNR heterodimers [Chun *et al* 2008; Sun *et al* 2011] in the presence of the non-activated APLNR, act to inhibit AT1R signalling [Sun *et al* 2011].

In preclinical and clinical studies, APLNR activation does not appear to potentiate the vascular effects of angiotensin II. In rodent veins pretreated with apelin, angiotensin II constriction effects are reduced [Gurzu *et al* 2006], whilst in whole animal studies apelin infusion abolishes the pressor effects of angiotensin II. There are limited data from clinical studies specifically assessing the interaction between apelin-APLNR and the renin-angiotensin system. Clinical studies have been performed in healthy volunteers, with no manipulation of the renin-angiotensin system, or in patients with chronic stable heart failure [Japp *et al* 2010]. Studies in patients with chronic stable heart failure, investigating the relationship between these two systems, are limited by the potential for chronic activation of the renin-angiotensin system and the fact that the patients are medicated with drugs that alter renin-angiotensin system activity.

It is essential to determine whether any receptor interaction exists functionally *in vivo* in man. We have undertaken clinical studies in both local and systemic

circulation to assess the interaction between these two systems following acute activation of the AT1R.

4.2.1 HYPOTHESIS

We hypothesise that the local and systemic cardiovascular actions of (Pyr^{1})apelin-13 will be reduced during acute angiotensin II infusion, through reduced availability of the APLNR, and this may even potentiate AT1R signalling.

4.3 METHODS

4.3.1 SUBJECTS

Twenty-four subjects were recruited, with two groups of 12 subjects allocated to local and systemic studies. Each subject attended on two occasions and at least one week apart. All subjects abstained from alcohol and caffeine for 24 hours and from food 4 hours prior to their study visit. All studies were performed in a quiet, temperature controlled room maintained at 22-24°C, with the subjects lying supine.

4.3.2 LOCAL VASCULAR STUDIES

Twelve subjects attended on one occasion, and local vascular responses to apelin were assessed during acute intrabrachial angiotensin II infusion (Figure 4.1). The dosage of angiotensin II (5-30 pmol/min) was up titrated until forearm blood flow was reduced by ~50%. Once sufficient and stable vasoconstriction was achieved, (Pyr^{1})apelin-13 (0.3, 1.0 and 3.0 nmol/min) (Genscript, NJ, USA) and sodium

nitroprusside (2, 4 and 8 µg/min) were co-infused with angiotensin II in a randomised double blind manner with each administration lasting for 6 minutes.

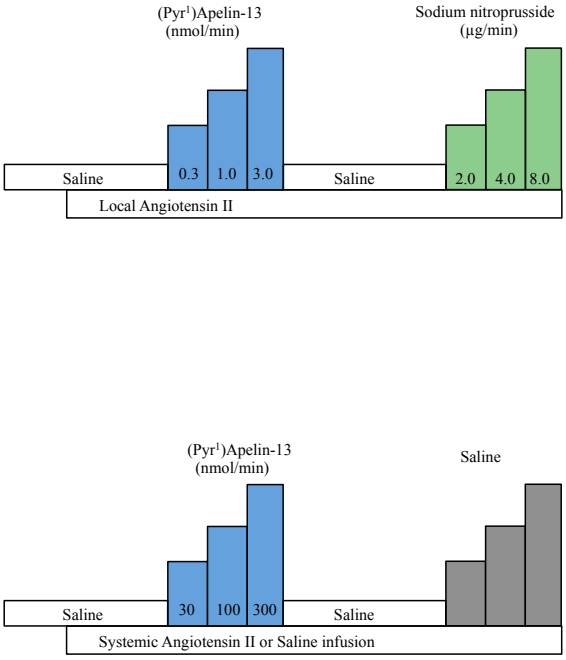

Figure 4.1. Study protocols.

4.3.3 SYSTEMIC VASCULAR STUDIES

In a double blind randomised crossover design, subjects received an infusion of intravenous angiotensin II at a subpressor dose (0.5 ng/kg/min) or a matched 0.9% saline placebo, as described previously [Ljungman *et al* 1983]. The subjects then received ascending doses of intravenous (Pyr^{1})apelin-13 (30, 100 and 300 nmol/min) (Genscript, NJ, USA) or a matched saline placebo, administered for 6 minutes at each dose and given in a double blind randomised crossover manner [Japp *et al* 2010]. The apelin and placebo administration sequence was randomised between subjects but was kept constant for each individual subject, in order to maintain a consistent order for both study visits.

4.3.4 DATA AND STATISTICAL ANALYSIS

Variables are reported as mean±SEM and analysed using repeated measures ANOVA with post-hoc Bonferroni corrections and paired two-tailed Student's *t*-test as appropriate (GraphPad Prism). Forearm blood flow was calculated from plethysmographic data, as described previously [Newby *et al* 1998; Japp *et al* 2008]. Mean arterial pressure was defined as the sum of the diastolic blood pressure and a third of the pulse pressure. Peripheral vascular resistance index was calculated as mean arterial pressure minus mean right atrial pressure, divided by cardiac index. Statistical significance was taken as $P < 0.05$. Based on power calculations derived from previous studies [Japp *et al* 2010] and a significance level of 5%, the sample sizes (n=12) will give 90% power of detecting the clinically meaningful differences of 0.7 mL/100 mL per minute and 0.6 L/min in forearm blood flow and cardiac output, respectively. We have previously described the influence of a range of factors

on regional and systemic vascular beds using sample sizes of 8 to 12 subjects. (Japp *et al* 2008, Japp *et al* 2010 , Newby *et al* 1996].

4.4 RESULTS

4.4.1 STUDY PARTICIPANTS

Healthy volunteers were male, aged 21±0 years and with a body mass index of 22±1 kg/m^2. All infusions were well tolerated, with no serious adverse events.

4.4.2 LOCAL VASCULAR ACTIONS OF APELIN DURING ACUTE RENIN-ANGIOTENSIN ACTIVATION

Local APLNR agonism was assessed with (Pyr^1)apelin-13 (Genscript). Local intra-arterial angiotensin II infusion caused a marked increase in local angiotensin II concentrations (95.6±16.0 *versus* 4.5±1.0 pg/mL; P <0.0001) that was accompanied by a reduction in forearm blood flow (from 2.5±0.4 to 1.3±0.3 mL/100 mL/min; P=0.001) in the infused forearm. In the local vasoconstriction setting, both (Pyr^1)apelin-13 (P=0.02) and sodium nitroprusside (P <0.0001) caused vasodilatation (Figure 4.2).

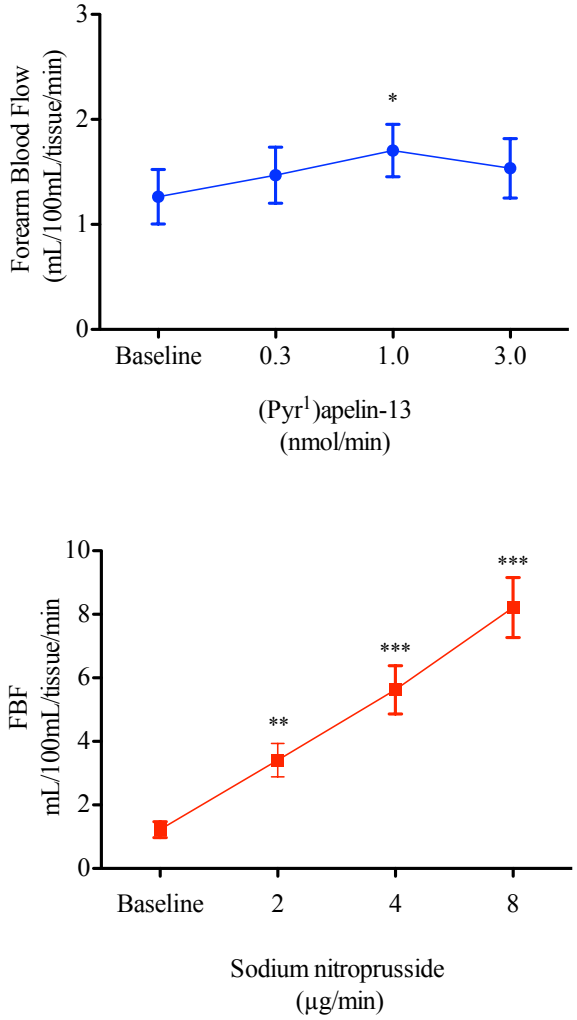

Figure 4.2. A) Forearm blood flow (mL/100mL/min) during intra-arterial (Pyr[1])apelin-13 (blue circles) following pre-constriction with angiotensin II. **P* <0.05, ***P* <0.01, ****P* <0.001. One-way ANOVA with a post-hoc Bonferroni test. **B)** Forearm blood flow (mL/100mL/min) during intra-arterial sodium nitroprusside (red squares) following pre-constriction with angiotensin II. **P* <0.05, ***P* <0.01, ****P* <0.001. One-way ANOVA with a post-hoc Bonferroni test.
ANOVA - analysis of variance.

4.4.3 SYSTEMIC VASCULAR ACTIONS OF APELIN DURING ACUTE RENIN-ANGIOTENSIN ACTIVATION

Systemic APLNR agonism was assessed with (Pyr^{1})apelin-13 (Genscript). Baseline haemodynamic data are presented in Table 4.1. No differences were observed, at baseline (prior to run-in infusion) and saline run-in infusion had no effect on systemic haemodynamics (data not shown). Intravenous angiotensin II infusion (0.5 ng/kg/min) increased systemic angiotensin II concentrations (from 3.7±0.4 to 7.3±0.8 pg/mL; P=0.001). Although it was our intention to administer a subpressor dose of angiotensin II [Ljungman *et al* 1983], we observed a small increase in both mean arterial pressure (from 82±1 to 86±1 mmHg; P=0.0014) and peripheral vascular resistance index (from 1420±80 to 1529±91 dynes.s/cm^{5}/m^{2}; P <0.0001), with a small reduction in cardiac index (from 4.6±0.2 to 4.4±0.2 L/min/m^{2}; P=0.0288) during angiotensin II infusion. Compared to placebo, angiotensin II infusion had no increasing effect on cardiac index or a reducing effect on mean arterial pressure or peripheral vascular resistance index observed during systemic (Pyr^{1})apelin-13 infusion (ANOVA; P >0.05, Figure 4.3). There appeared to be an initial increase in heart rate following apelin infusion (ANOVA; P <0.02) during angiotensin II co-infusion, although this was not significant for the placebo infusion.

TABLE 4.1 Baseline and post run-in systemic haemodynamics

	Angiotensin II infusion baseline	Angiotensin II infusion run-in	Saline infusion
Cardiac Index L/min/m^2	4.6±0.2	4.4±0.2	4.4±0.2
Heart Rate beats/min	57.4±1.9	58.9±2.1	55.4±2.3
Mean Arterial Pressure mmHg	82.4±1.1	86.2±4**	82.4±1.6
Peripheral Vascular Resistance Index dynes.s/cm^5/m^2	1420±80.0	1534±97	1479±870

Paired Student's *t*-test, angiotensin baseline *versus* run-in.
**P* <0.05, ***P* <0.01, ****P* <0.001.

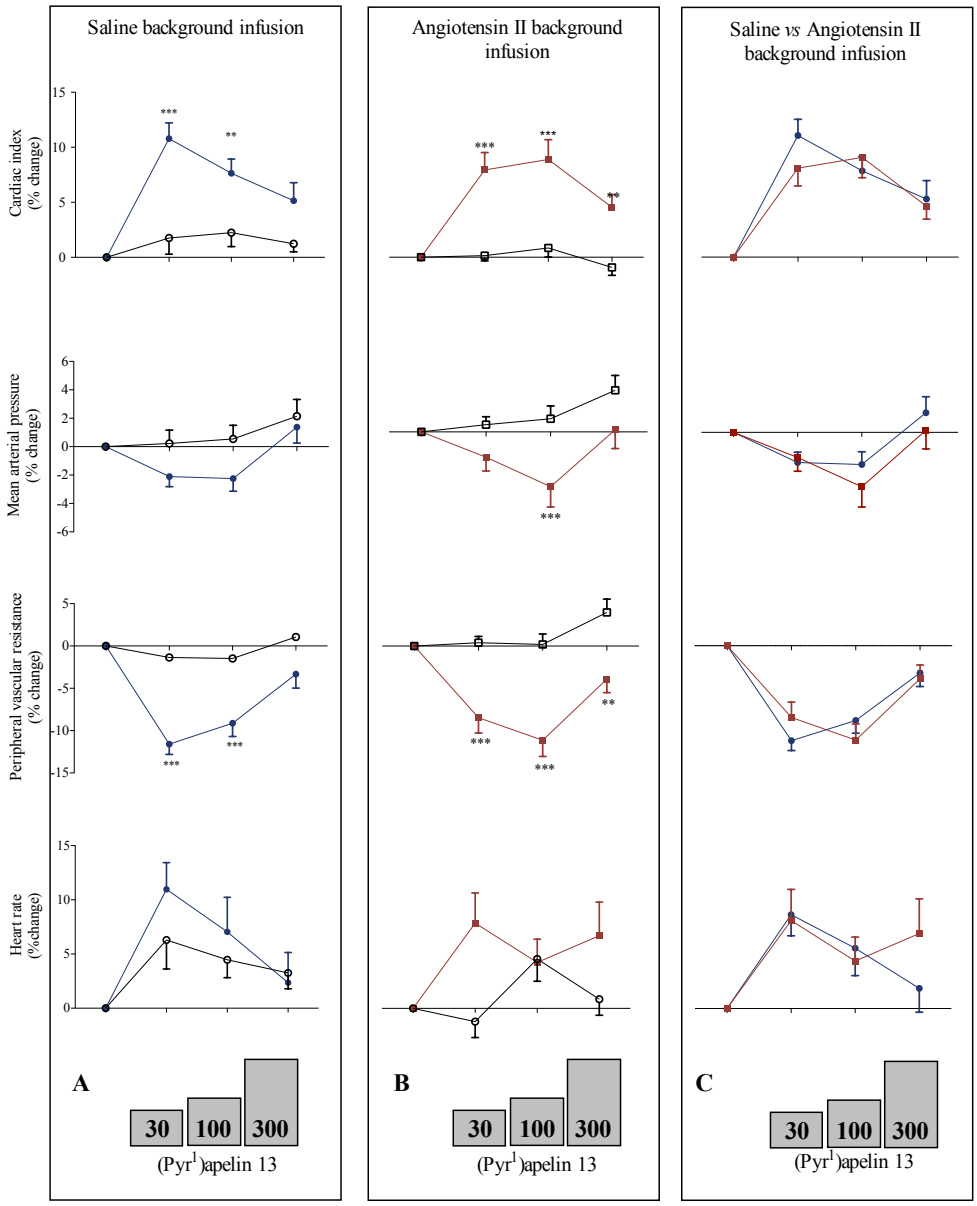

Figure 4.3. A) Background saline infusion; percentage change in cardiac index, mean arterial pressure, peripheral vascular resistance index and heart rate during systemic infusion of (Pyr[1])apelin-13 (blue circles, closed) or saline (black circles open) **P* <0.05, ***P* <0.01, ****P* <0.001. Two-way ANOVA with post-hoc Bonferroni test. **B)** Background angiotensin II infusion; percentage change in cardiac index, mean arterial pressure, peripheral vascular resistance index and heart rate during systemic infusion of (Pyr[1])apelin-13 (red squares, closed) or saline (black squares, open). **P* <0.05, ***P* <0.01, ****P* <0.001. Two-way ANOVA with post-hoc Bonferroni test. **C)** Comparison of background infusions, saline or angiotensin II; percentage change in cardiac index, mean arterial pressure, peripheral vascular resistance index and heart rate during systemic infusion of (Pyr[1])apelin-13 during placebo infusion (blue circles, closed) or angiotensin II infusion (red squares, closed). **P* <0.05, ***P* <0.01, ****P* <0.001. Two-way ANOVA with post-hoc Bonferroni test.
ANOVA - analysis of variance.

4.5 DISCUSSION

In these studies we have shown for the first time in man that the local and systemic cardiovascular actions of acute (Pyr^{1})apelin-13 infusions are unaffected by acute angiotensin II elevation and APLNR signalling persists in the presence of AT1R activation.

Angiotensin II infusions resulted in 21-fold and 2-fold increases in local and systemic plasma angiotensin II concentrations, respectively. During local vascular studies, local forearm blood flow was reduced by ~50%, and in systemic studies, although the protocol was designed to administer a subpressor dose of angiotensin II, pressor effects during systemic infusion were evident. Whilst the blood pressure elevations observed were of questionable biological significance, the changes in mean arterial pressure were of statistical significance. In all phases of the study, we successfully achieved a range of renin-angiotensin systemic activation, and we do not believe that the lack of effect is due to a failure to augment plasma angiotensin II concentrations.

Accumulating evidence suggests acute interactions between the apelin-APLNR system and the renin-angiotensin system and, in particular, the interaction between the AT1R and the APLNR [Chun *et al* 2008; Sun *et al* 2011]. In cellular models, the AT1R has been shown to form heterodimers with other G protein-coupled receptors [AbdAlla *et al* 2000; AbdAlla *et al* 2001; AbdAlla *et al* 2005; Li *et al* 2012]. These interactions are reported to have important functional consequences in preclinical

studies as they alter the activity of intracellular signalling cascades and receptor metabolism. Formation of heterodimers can result in exaggerated angiotensin II effects, as seen with the angiotensin type 1 receptor-bradykinin B2 heterodimer complexes [AbdAlla *et al* 2000; AbdAlla *et al* 2005], or dampened responses, as with angiotensin type 2 receptors [AbdAlla *et al* 2001].

Preclinical studies investigating the functional consequences of APLNR-AT1R heterodimers have shown that this complex inhibits angiotensin II actions. Sun *et al* [2011] reported that in the presence of the inactive APLNR receptor, angiotensin II signalling was inhibited as a result of heterodimer formation, and with the addition of apelin to this cellular model, angiotensin II signals were enhanced. This suggests that ligand binding to APLNR prevents dimerisation and angiotensin II inhibition, which implies that the APLNR in its basal form functions to antagonise AT1R signalling. In this study there was no evidence of a potentiated angiotensin II response with (Pyr^{1})apelin-13 infusion. Whilst Chun *et al* [2008], who first reported the formation of an APLNR-AT1R heterodimer, report the inhibition of angiotensin II-mediated signals in the presence of the APLNR, there was no enhancement of angiotensin II effects in the presence of activated APLNR.

It must be acknowledged that evidence of heterodimerisation is limited to preclinical studies and often in cellular constructs, which may not accurately reflect receptor interaction in tissues *in vivo* in humans. Furthermore, this phenomenon does not appear to be universal, with studies reporting no evidence of angiotensin type 1 receptor-bradykinin B2 receptor heterodimer complexes in a

range of models, which questions the functional relevance of this suggested interaction [Hansen *et al* 2009].

4.5.1 LIMITATIONS

No evidence of an interaction between these two systems has been identified in the acute setting. From preclinical studies we could have predicted that co-infusion of angiotensin II and (Pyr^{1})apelin-13 may result in reduced efficacy of (Pyr^{1})apelin-13, or even increase the efficacy of angiotensin II. We do not believe the study was underpowered, however, it is possible that an interaction is present but below the limits of detection by the techniques used; alternatively, it may be sufficiently small that more individuals would need to be studied.

No control vasoconstrictor was used in this study protocol and alternative vasoconstrictor, such as noradrenaline, would allow some inference to a general vasodilator action or a more specific function to reverse angiotensin II mediated vasoconstriction.

These studies were undertaken to assess acute interactions that may reflect direct receptor interactions. At present no methods are available to assess examination of receptor complexes and interaction *in vivo*. Furthermore, if heterodimer formation is of biological significance, it is conceivable that multiple dynamic receptor interactions exist which have not been fully characterised in preclinical models.

These studies have been performed in healthy individuals, free from cardiovascular disease and not receiving any regular medication. The results of the study may not be universal and there may be important interactions between these two systems in diseases such as heart failure.

In summary, acute local and systemic actions of (Pyr^{1})apelin-13 are preserved in the presence of acute angiotensin II infusion. This suggests that the signalling of the APLNR is unaffected by angiotensin II, and that AT1R signalling is unaffected by (Pyr^{1})apelin-13. These data do not confirm any interactions between these two systems during the investigated period; however, we cannot exclude the possibility that an interaction does exist or that these two systems may have important interactions in different settings.

CHAPTER 5

INVESTIGATING THE INOTROPIC POTENTIAL OF APELIN IN CHRONIC STABLE HEART FAILURE

Japp AG, Cruden NL, **Barnes GD** *et al.*
Acute cardiovascular effects of apelin in humans: potential role in patients with chronic heart failure.
Circulation 2010;**121**(16):1818-1827.

5.1 SUMMARY

Introduction In preclinical studies the apelin-APLNR systems maintain cardiac contractile function, whilst exogenous apelin is a potent inotrope. Furthermore in heart failure models the apelin-APLNR system is downregulated suggesting a pathophysiological role, but raises concerns of tachyphylaxis. The aim of this study was to assess the safety and cardiovascular effects of systemic (Pyr^{1})apelin-13 infusion.

Methods Eight patients with chronic stable heart failure attended on two visits, at least one week apart. Cardiac index, systemic vascular resistance index, heart rate and mean arterial pressure assessed by thoracic electrical bioimpedance and a semi-automated sphygmomanometer respectively during systemic (Pyr^{1})apelin-13 infusion (30, 100 and 300 nmol/min) or placebo (0.9% saline) in a randomised double blinded crossover design.

Results Acute (Pyr^{1})apelin-13 infusion resulted in increased cardiac output and a reduction in mean arterial pressure during acutely systemic (Pyr^{1})apelin-13 infusion (P <0.05).

Conclusion The cardiovascular effects of exogenous (Pyr^{1})apelin-13 are preserved in patients with chronic stable heart failure. These data support the role of APLNR agonism in chronic stable heart failure, and suggest that there is sufficient

myocardial APLNR density to evoke a meaningful response. Longer term studies are required to fully assess the therapeutic potential of this system in heart failure.

5.2 INTRODUCTION

The apelin receptor is expressed in high quantities in the myocardium [Kleinz and Davenport 2004] and functions as a positive inotrope. In isolated cardiomyocytes, apelin is reported to be the most potent inotrope studied *in vitro* and is effective at subnanomolar concentrations [Farkasfalvi *et al* 2007]. Similarly, in paced atrial strips, there is a dose-dependent contractile effect at subnanomolar concentrations [Maguire *et al* 2009]. In perfused hearts [Berry *et al* 2004] and whole animal studies, APLNR agonism mediates positive inotropic effects, independent of cardiac loading conditions and without inducing left ventricular hypertrophy [Ashley *et al* 2005]. Initial clinical studies in healthy volunteers are in agreement with preclinical studies, and exogenous (Pyr^{1})apelin-13 infusion mediates positive inotropism [Japp *et al* 2008].

The apelin-APLNR system has an important role in maintaining normal cardiac function. Whilst apelin-deficient mice have normal cardiovascular development, they progress to heart failure with age and increased afterload [Kuba *et al* 2007]. Furthermore, apelin-deficient rodents have a reduced fractional shortening and end-systolic pressure volume relationship at baseline, along with impaired ventricular performance during stress. Equally, exercise ability of rodents in reduced in either rodents deficient in apelin or the APLNR [Charo *et al* 2009].

Given the cardiovascular actions of exogenous apelin, and the requirement of the apelin-APLNR system to maintain normal cardiac function, the apelin-APLNR system has been investigated in ventricular dysfunction, in both preclinical and clinical studies. In cardiomyocytes exposed to repetitive stretch, analogous to volume overload in ventricular dysfunction, there is rapid downregulation of APLNR expression [Szokodi *et al* 2002]. Additionally, in hypertensive models of heart failure, as left ventricular dysfunction ensues there is downregulation of both cardiac apelin and APLNR expression [Iwanaga *et al* 2006].

In man, there are limited data regarding cardiac apelin expression. However, in patients with left ventricular dysfunction of idiopathic aetiology, APLNR downregulation is evident [Földes *et al* 2003; Pitkin *et al* 2010]. Furthermore, in patients undergoing heart transplantation, who are treated with left ventricular assist devices prior to surgery, apelin is the most upregulated gene in the myocardium [Chen *et al* 2003].

The utility of circulating apelin as a biomarker has been assessed in patients with ventricular dysfunction. An initial increase in plasma apelin circulation has been reported in early heart failure, suggesting a compensatory role, with a reduction in more severe heart failure, suggesting the requirement of apelin to maintain normal cardiac function [Chen *et al* 2003]. However, others have failed to demonstrate any relationship between the extent of ventricular dysfunction and plasma concentration beyond a reduction in heart failure [Chong *et al* 2006].

Taken together, these data support a role for the apelin-APLNR system in maintaining normal cardiac function and show downregulation in heart failure, suggesting a pathophysiological role. Characterising the action of apelin in heart failure is essential to explore any therapeutic benefit of this system.

5.2.1 HYPOTHESIS

Systemic infusion of (Pyr^{1})apelin-13 in patients with chronic stable heart failure will result in an increase in cardiac output and reduction in both mean arterial pressure and systemic vascular resistance.

5.3 METHODS

Eight patients with chronic stable heart failure were recruited and attended on one occasion. Patients were eligible for inclusion if they had chronic stable heart failure and were in NYHA class II-IV, receiving maximally tolerated doses of heart failure medication for at least 3 months and had evidence of left ventricular dysfunction on echocardiography examination (left ventricular end-diastolic diameter >5.5 cm, left ventricular ejection fraction <40% or fractional shortening <20%). Patients were excluded if they fulfilled any of the following exclusion criteria: valvular heart disease, significant renal or hepatic failure, women of child-bearing potential not on adequate contraception or previous malignant arrhythmias. On the day of the study, the patients withheld their usual medication and were asked to abstain from alcohol

and caffeine for 24 hours and food for 4 hours. All studies were performed in a quiet, temperature controlled room.

5.3.1 HAEMODYNAMIC STUDIES

17-gauge cannulas were inserted into the large antecubital fossa veins in each arm, allowing for drug infusion and venous sampling. Blood pressure and heart rate were recorded with a semi-automated sphygmomanometer while mean arterial pressure was calculated as diastolic blood pressure plus one-third pulse pressure. Cardiac output was measured using HOTMAN thoracic bioimpedance and corrected for body surface area to derive cardiac index. Peripheral vascular resistance index was calculated as mean arterial pressure minus mean right atrial pressure, divided by the cardiac index. The electrocardiograph was recorded continuously during each study (Figure 5.1). Cardiac index, peripheral vascular index, mean arterial blood pressure and heart rate were measured during systemic (Pyr^{1})apelin-13 infusion (30, 100, 300 nmol/min) (Clinalfa AG, Läufelfingen, Switzerland) or placebo infusion, administered for 6 minutes. (Pyr^{1})apelin-13 infusion and placebo infusions were separated by a 30-minute wash out period.

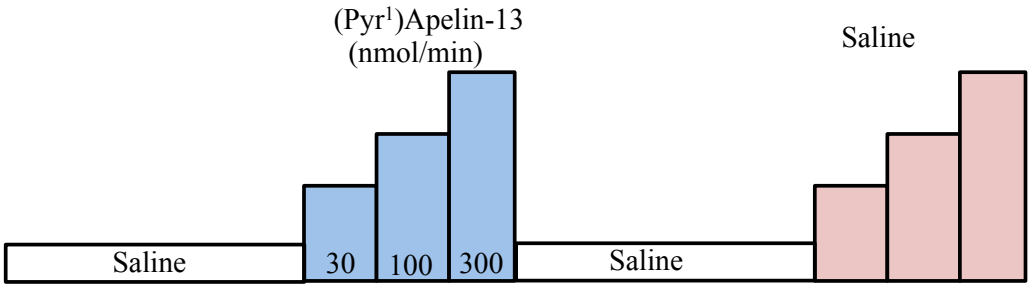

Figure 5.1. Study protocol.

5.3.2 DATA AND STATISTICAL ANALYSIS

Variables are reported as mean±SEM and analysed using repeated measures ANOVA with post-hoc Bonferroni corrections and paired two-tailed Student's *t*-test as appropriate (GraphPad Prism). Mean arterial pressure was defined as the sum of the diastolic blood pressure and a third of the pulse pressure. Peripheral vascular resistance index was calculated as mean arterial pressure minus mean right atrial pressure, divided by the cardiac index. Statistical significance was taken as $P < 0.05$. For 80% power at 2-sided P 0.05 and based on our previously published data a sample size of 8 selected to detect differences in cardiac output of 0.56 L/min.

5.4 RESULTS

5.4.1 PATIENT CHARACTERISTICS

Table 5.1 shows baseline characteristics and current therapy. Patients were all male with poor contractile function assessed by echocardiography. These patients were maintained evidence based therapies, with only one patient not prescribed renin-angiotensin inhibition/antagonism.

TABLE 5.1 Baseline characteristics of patients with chronic stable heart failure

Baseline Characteristics	
Age (Yrs)	55±4
Sex (Male/Female)	8/0
Aetiology (Ischaemic/Idiopathic)	5/3
NYHA II/III/IV	4/4/C
Echocardiographic Measures	
Left Ventricular End-diastolic Diameter (cm)	6.5±0.5
Left Ventricular Ejection Fraction	19±2%
Concomitant Therapy	
β-blockade	8/8
Angiotensin-converting Enzyme Inhibitor/ Angiotensin Receptor Blocker	7/8
Aldosterone Receptor Antagonist	4/8

5.4.2 SYSTEMIC (PYR[1])APELIN-13 INFUSION: SAFETY

No adverse events were observed during apelin administration, and apelin infusion was tolerated in all subjects. No arrhythmias were induced throughout infusion.

5.4.3 SYSTEMIC (PYR[1])APELIN-13 INFUSION: EFFICACY

Systemic APLNR agonism was assessed with (Pyr[1])apelin-13 (Clinalfa). Intravenous (Pyr[1])apelin-13 infusion increased cardiac index (P <0.01 for both), reduced mean arterial pressure and reduced peripheral vascular resistance index (P <0.001 for both) in patients with chronic stable heart failure. No linear dose response was evident, and a rapid plateau was recorded (Figure 5.2).

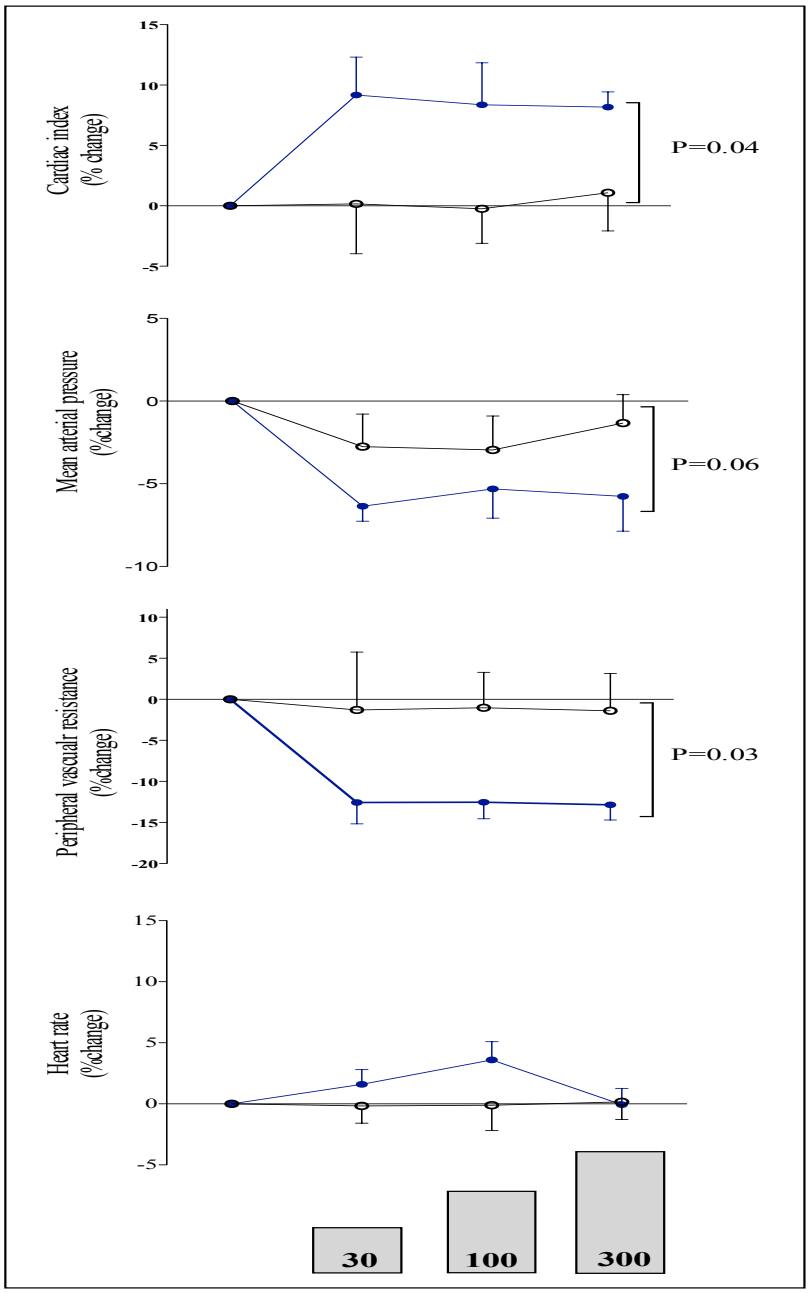

Figure 5.2. Percentage change from baseline in cardiac index, mean arterial pressure, peripheral vascular resistance and heart rate during intravenous (Pyr[1])apelin-13 (blue circles, closed) or placebo (black circles, open) (blue squares, bottom). Grey bars show (Pyr[1])apelin-13 infusions, mmol/min.*P <0.05, **P <0.01, ***P <0.001. Two-way ANOVA with post-hoc Bonferroni test. ANOVA - analysis of variance.

5.5 DISCUSSION

This is the first study administering systemic (Pyr[1])apelin-13 to patients with chronic stable heart failure, and it resulted in an increased cardiac index and reduced afterload. These data are in keeping with preclinical models and clinical studies in healthy volunteers.

5.5.1 SAFETY

All studies of (Pyr[1])apelin-13 were well tolerated, with no adverse effects recorded. Of particular importance, no arrhythmias were recorded during these studies.

5.5.2 EFFICACY

In these studies, (Pyr[1])apelin-13 increased cardiac index by around 10% in patients with chronic stable heart. These results are encouraging and suggest that the APLNR may be a promising target in ventricular dysfunction.

There are data supporting downregulation of the apelin-APLNR system in heart failure [Szokodi *et al* 2002; Chen *et al* 2003; Földes *et al* 2003; Chong *et al* 2006; Iwanaga *et al* 2006; Francia *et al* 2007; Pitkin *et al* 2010]. In isolated cardiomyocytes exposed to repeated stretching over 24 hours, analogous to dilated heart failure, the APLNR is downregulated [Szokodi *et al* 2002]. In whole animal hypertensive heart failure models the apelin-APLNR system becomes downregulated as heart failure

ensues [Iwanaga *et al* 2006]. There are limited data in man regarding myocardial apelin-APLNR expression, but the APLNR is downregulated in dilated cardiomyopathy [Földes *et al* 2003; Pitkin *et al* 2010]. Furthermore, plasma concentrations of apelin are reduced in patients with heart failure [Chen *et al* 2003; Földes *et al* 2003; Chong *et al* 2006; Francia *et al* 2007].

It is proposed that downregulation in the apelin-APLNR system may contribute to ventricular dysfunction although, this raises concerns regarding insufficient APLNR density to evoke a signal. In this study we have shown that during acute infusion, exogenous (Pyr^{1})apelin-13 increases cardiac output in patients with chronic stable heart failure. The echocardiographic features of this cohort are consistent with severe ventricular dysfunction with a mean ejection fraction of around 20% and dilated end-diastolic diameters. It is likely that myocardial APLNR expression will be reduced in this cohort, and the effects that have been observed are representative of patients with heart failure. Furthermore, the patients recruited for this study were maintained on evidence-based therapies, and therefore the improvement in systemic haemodynamics during (Pyr^{1})apelin-13 infusion represents an incremental benefit.

The underlying mechanism mediating the increased cardiac output observed in this study has not been determined. It is possible that there is a direct inotropic effect on the myocardium, however this may be a response to vasodilatation. A reduction in afterload will make some contribution, but preclinical data show direct effects of apelin on the myocardium [Szokodi *et al* 2002; Farkasfalvi *et al* 2007], and in whole animals increased cardiac output is seen during fixed preload [Berry *et al* 2004]. In

patient studies, performing pressure volume loads to assess myocardial contractility would be both technically and ethically challenging.

The dose-response relationship shows rapid plateau suggesting that APLNR signalling may be fatigued easily or that it quickly reaches maximal response. Further work is therefore required to assess the utility of exogenous (Pyr^{1})apelin-13 with prolonged APLNR activation. Although presently the acute effects are encouraging, it is essential to understand whether these effects are restricted to the acute setting or if more sustained responses are possible. There is no oral preparation available at present, which limits the study of chronic APLNR agonism, thus making an assessment on ventricular remodelling or prognosis impossible. Preclinical studies support a role for apelin in preventing cardiac fibrosis [Siddiquee *et al* 2011] and renin-angiotensin inhibition [Sun *et al* 2011]; consequently, the true value of the apelin-APLNR system may extend beyond its haemodynamic properties.

5.5.3 LIMITATIONS

The duration of infusion was brief, at around 20 minutes in total, and the number of patients included was small. Whilst the duration of infusion is appropriate in investigational studies, it represents a brief timescale in the context of heart failure. Therefore, these data are not informative when considering long term therapeutic benefits. Encouragingly, though, no adverse events were recorded during this study, however using positive inotropes in heart failure may promote arrhythmias and given the small number of patients studied, we cannot exclude this concern.

In conclusion, this study is the first to profile the cardiovascular actions of exogenous (Pyr^{1})apelin-13 in patients with chronic stable heart failure. We have demonstrated that acute infusions are safe, with no adverse events recorded, and APLNR stimulation mediates increased cardiac output. The apelin-APLNR system merits further investigation in heart failure.

CHAPTER 6

INVESTIGATING THE CARDIOVASCULAR EFFECTS OF PROLONGED (PYR[1])APELIN-13 INFUSION IN HEALTHY VOLUNTEERS AND PATIENTS WITH CHRONIC STABLE HEART FAILURE

Barnes GD, Alam S, Carter G *et al*.
Sustained cardiovascular actions of APJ agonism during renin-angiotensin system activation and in patients with heart failure.
Circ Heart Fail 2013;**6**(3):482-491.

6.1 SUMMARY

Introduction In humans, plasma apelin concentrations and ventricular APLNR expression are reduced in heart failure. Apelin infusion has a favourable cardiovascular profile, although clinical trials have currently been limited to short 15-minute infusions and there are major concerns regarding tachyphylaxis. The aim of this study was to assess the systemic cardiovascular response to (Pyr^{1})apelin-13 infusion over 6 hours in healthy volunteers and patients with chronic stable heart failure.

Methods Twelve healthy volunteers and 12 patients with chronic stable heart failure were recruited and received systemic (Pyr^{1})apelin-13 (30 nmol/min) infusion or a placebo in a double blinded crossover design. Cardiac index, peripheral vascular resistance index, heart rate and mean arterial pressure were assessed using thoracic electrical bioimpedance and semi-automated sphygmomanometer. Left ventricular dimensions were assessed in patients with chronic stable heart failure at 15-minute intervals with echocardiography.

Results In both healthy volunteers and patients with chronic stable heart failure, systemic (Pyr^{1})apelin-13 infusion resulted in increased cardiac output throughout the first hour and was sustained over 6 hours. In patients with chronic stable heart failure, improvements in contractile indices were seen with echocardiography. No adverse events were seen in either the healthy volunteers or patients during (Pyr^{1})apelin-13

infusion. This study provides important information regarding the APLNR as a therapeutic target.

Conclusion (Pyr^1)apelin-13 infusion produced a sustained effect during the infusion process, with no evidence of tachyphylaxis, suggesting that the APLNR holds major promise for the chronic treatment of left ventricular failure.

6.2 INTRODUCTION

First identified in 1993 [O'Dowd *et al* 1993], the APLNR is widely expressed throughout the body and, in particular, on the endothelium, vascular smooth muscle cells and cardiomyocytes [Hosoya *et al* 2000; Kleinz *et al* 2005; Farkasfalvi *et al* 2007]. It is a G protein-coupled receptor which remained orphaned until its endogenous ligand apelin was discovered in 1998 [Tatemoto *et al* 1998]. Although various apelin peptide fragments exist, the pyroglutamated 13 amino acid form of apelin, (Pyr^1)apelin-13, is the most potent and abundant form in cardiac tissue [Maguire *et al* 2009] and stimulates the APLNR to cause vasodilatation [Lee *et al* 2000; Tatemoto *et al* 2001] and positive inotropism [Szokodi *et al* 2002; Farkasfalvi *et al* 2007].

The apelin-APLNR system has an important role in maintaining cardiac function. Apelin-deficient animal models exhibit reduced cardiac contractility through ageing and severe cardiac failure in response to increased afterload [Kuba *et al* 2007]. Furthermore, apelin-deficient rodents have impaired basal ventricular function and

exercise tolerance [Charo *et al* 2009]. Expression of the APLNR is reduced in isolated cardiomyocytes subjected to mechanical stretch [Szokodi *et al* 2002] and animal models of heart failure [Iwanaga *et al* 2006]. In man, ventricular APLNR expression is reduced in heart failure [Földes *et al* 2003; Pitkin *et al* 2010], and there is therefore a concern that the effects of APLNR agonism may be abrogated or ineffective in patients with heart failure. Furthermore, in keeping with many G protein-coupled receptors [Leonard and Gulati 2009], there remains the potential for prolonged APLNR agonism by apelin to cause rapid desensitisation and tachyphylaxis, which in turn would limit the clinical utility of any potential therapeutic strategy targeted at chronic APLNR agonism. The dose response curve to APLNR agonism rapidly plateaus, which again raises concern regarding prolonged agonism.

The role of exogenous apelin on myocardial contractility has been investigated in both preclinical and clinical models. In isolated myocytes apelin is a potent inotrope at subnanomolar concentrations [Szokodi *et al* 2002], and this inotropic effect is evident in both isolated perfused hearts and animal models. In man, systemic apelin infusion produces a modest increase in cardiac output [Japp *et al* 2010], whilst in heart failure, exogenous apelin increases cardiac output in preclinical models of heart failure [Berry *et al* 2004; Dai *et al* 2006; Atluri *et al* 2007] and in patients with chronic stable heart failure [Japp *et al* 2010]. There are limited data assessing prolonged APLNR agonism. One preclinical study demonstrated continued efficacy during a 2-week infusion [Ashley *et al* 2005], without inducing left ventricular

hypertrophy. However, clinical studies in heart failure are restricted to acute infusions [Japp *et al* 2010].

The aims of this study were to assess the cardiovascular actions of (Pyr^1)apelin-13 during a sustained, prolonged infusion, in healthy volunteers and patients with chronic stable heart failure.

6.2.1 HYPOTHESIS

Prolonged systemic infusion of (Pyr^1)apelin-13 in healthy volunteers and patients with chronic stable heart failure will result in sustained increase in cardiac output and reduce mean arterial pressure and systemic vascular resistance.

6.3 METHODS

6.3.1 HEALTHY VOLUNTEERS

Twelve healthy volunteers attended on two occasions, at least one week apart, having been randomised in a double blinded crossover design to a systemic (Pyr^1)apelin-13 30 nmol/min (Genscript, NJ, USA) or matched saline placebo infusion (Figure 6.1). All studies were performed with the approval of the local Ethics Research Committee, in accordance with the Declaration of Helsinki and the written consent of all volunteers. Subjects were excluded if they were receiving any regular medication, had any significant past medical history, were current smokers or had participated in research studies within 3 months of enrolment. One participant was

enrolled and subsequently withdrawn, as he was found during echocardiographic examination to have a bicuspid aortic valve.

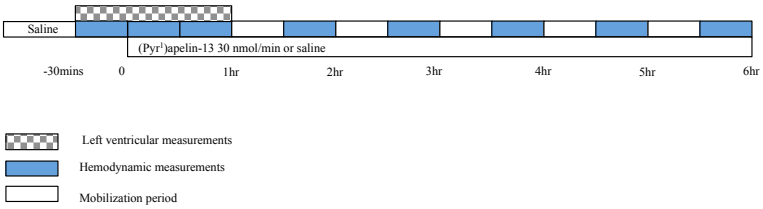

Figure 6. 1. Study protocol.

6.3.2 PATIENTS WITH CHRONIC STABLE HEART FAILURE

Twelve subjects with chronic stable heart failure attended on two occasions, at least one week apart, having been randomised in a double blinded crossover design to a systemic (Pyr^1)apelin-13 (30 nmol/min) or matched saline placebo infusion (Figure 6.1). Patients were eligible for inclusion if they had chronic stable heart failure and were in NYHA class II-IV, receiving maximally tolerated doses of heart failure medication for at least 3 months and had evidence of left ventricular dysfunction on echocardiography examination (left ventricular end-diastolic diameter >5.5 cm, left ventricular ejection fraction <40% or fractional shortening <20%). Patients were excluded if they fulfilled any of the following exclusion criteria: valvular heart disease, significant renal or hepatic failure, women of child-bearing potential not on adequate contraception or previous malignant arrhythmias. Case notes for all patients under the care of the community heart failure nurse

specialists at the Royal Infirmary of Edinburgh were reviewed. From this review, 38 patients were contacted and received patient information sheets, in order to recruit 12 patients to the study.

All studies were performed with the approval of the local Ethics Research Committee, in accordance with the Declaration of Helsinki and the written consent of all volunteers. All studies were performed in a quiet, temperature controlled room maintained at 22-24°C, with the subjects lying supine.

Cardiac index, peripheral vascular resistance, mean arterial pressure and heart rate were measured every 5 minutes during a 0.9% saline-using thoracic impedance cardiography and a semi-automated non-invasive sphygmomanometer, as previously described [Thomas 1992; Japp *et al* 2010]. Once all readings were within 10% of a rolling average, and only after a minimum 30-minute run-in infusion, cardiac index, peripheral vascular resistance, mean arterial pressure and heart rate were measured during systemic (Pyr^{1})apelin-13 infusion (30 nmol/min) or placebo administration for 6 hours. Infusions were administered in a randomised double blinded manner. Systemic (Pyr^{1})apelin-13 dosage was determined from previous studies within our group [Japp *et al* 2010]. Left ventricular end-systolic and diastolic measurements were assessed at baseline and at 15-minute intervals throughout the first hour of infusion in patient with suitable images (n=8), with fractional shortening and ventricular ejection fraction (Teichholz method) derived from these measurements.

In both health volunteer and patients with heart failure APLNR agonism was investigated with pharmaceutical grade (Pyr^{1})apelin-13 from Genscript, NJ, USA).

6.3.3 DATA AND STATISTICAL ANALYSIS

Variables are reported as mean±SEM and analysed using repeated measures ANOVA with post-hoc Bonferroni corrections and paired two-tailed Student's *t* test as appropriate (GraphPad Prism). Mean arterial pressure was defined as the sum of the diastolic blood pressure and a third of the pulse pressure. Peripheral vascular resistance index was calculated as mean arterial pressure minus mean right atrial pressure, divided by cardiac index. Statistical significance was taken as $P < 0.05$. Based on power calculations derived from previous studies [Japp *et al* 2010] and a significance level of 5%, the sample sizes (n=12) will give 90% power of detecting the clinically meaningful differences of 0.6 L/min in cardiac output. We have previously described the influence of a range of factors on regional and systemic vascular beds using sample sizes of 8 to 12 subjects. (Japp *et al* 2008, Japp *et al* 2010 , Newby *et al* 1996].

6.4 RESULTS

6.4.1 SAFETY OF PROLONGED (PYR^{1})APELIN-13 INFUSIONS

No serious adverse effects occurred during prolonged infusion in either the healthy volunteers or in patients with chronic stable heart failure. No arrhythmias were recorded in either cohort. Two studies were stopped prematurely in the heart failure group, one

due to a pre-existing chronic back problem and the other due to uncontrolled hypertension, which was long-standing.

6.4.2 PROLONGED (PYR[1])APELIN-13 INFUSIONS IN HEALTHY VOLUNTEERS

Systemic APLNR agonism was assessed with (Pyr[1])apelin-13 (Genscript). Baseline haemodynamics are presented in Table 6.1. Compared to the placebo, (Pyr[1])apelin-13 caused an increase in cardiac index during the first hour (ANOVA; P <0.0001), which was sustained throughout the 6-hour infusion (ANOVA; P <0.0001). There was an apparent increase in heart rate during the first hour (ANOVA; P=0.11), which became statistically significant during the remaining 5 hours of infusion (ANOVA; P=0.0002). Peripheral vascular resistance index was reduced during the first hour of infusion (ANOVA; P <0.0001) but not sustained to the end of the infusion (ANOVA; P=0.12). Mean arterial pressure was unchanged throughout the (Pyr[1])apelin-13 infusion (Figure 6.2)

TABLE 6.1 Baseline haemodynamic data

	(Pyr[1])apelin-13	**Placebo**
Cardiac Index L/min/m^2	4.4±0.3	4.4±0.3
Heart Rate beats/min	61.8±2,8	59.7±2.5
Systolic Blood Pressure mmHg	123.1±1.7	126.8±2.5
Diastolic Blood Pressure mmHg	74.5±1.4	77.4±2.2
Mean Arterial Pressure mmHg	90.7±1.3	92.1±1.2

Peripheral Vascular Resistance Index dynes.s/cm^5/m^2	1584±98.9	1577±96.4

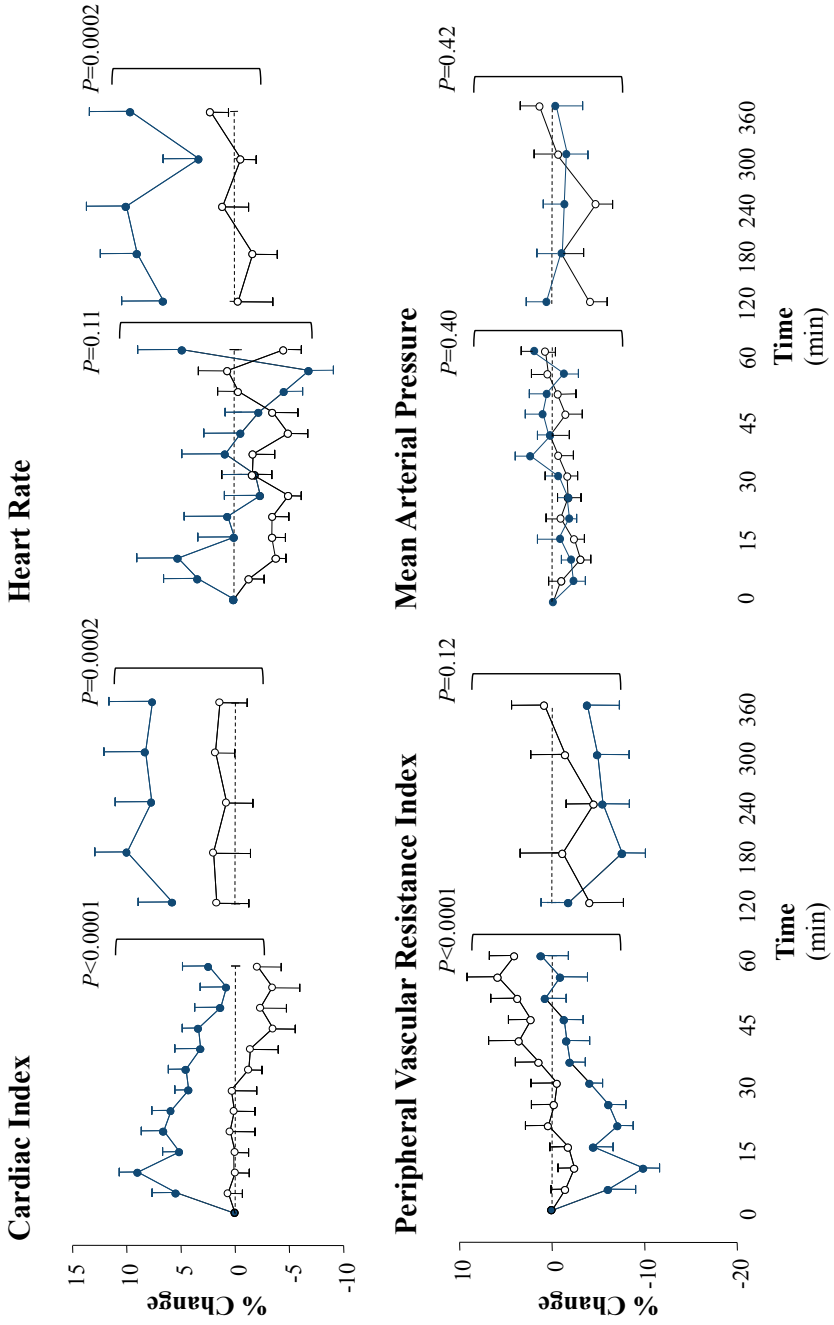

Figure 6.2. Percentage change in cardiac index, mean arterial pressure, peripheral vascular resistance index and heart rate during systemic infusion of (Pyr^1)apelin-13 (blue circles, closed) or

saline (black circles, open). *P <0.05, **P <0.01, ***P <0.001. One-way ANOVA with a post-hoc Bonferroni test. ANOVA - analysis of variance.

6.4.3 PATIENTS WITH CHRONIC STABLE HEART FAILURE

Systemic APLNR agonism was assessed with (Pyr[1])apelin-13 (Genscript). Patient characteristics and baseline haemodynamic data are presented in Tables 6.2 and Table 6.3, respectively. Intravenous (Pyr[1])apelin-13 infusion increased cardiac index during the first hour (ANOVA; P=0.003), and this was sustained throughout the 6-hour infusion (ANOVA; P=0.0003). Both mean arterial pressure and the peripheral vascular resistance index were reduced during the initial hour of infusion (ANOVA; P=0.008 and P=0.0001, respectively), and this was maintained during the 6-hour infusion (ANOVA; P=0.007 and P=0.002, respectively). There was an apparent trend for an increase in heart rate during the first hour of infusion (ANOVA; P=0.051), although this did not persist or reach statistical significance during the 6-hour infusion (ANOVA; P=0.42: Figure 6.3). Fractional shortening and left ventricular ejection fraction were increased following apelin infusion (ANOVA; P <0.0001: Figure 6.4).

TABLE 6.2 Baseline characteristics of patients with chronic heart failure

Baseline Characteristics		
Age (Yrs)		64±3
Sex (Male/Female)		8/4
Aetiology (Ischaemic/Idiopathic)		8/4
NYHA	II/III/IV	7/ 5/0
Left Ventricular Function	Severe (Ejection Fraction <20%)	9/12
	Moderate (Ejection Fraction 20-30%)	3/12
Echocardiographic Measures*		
Left Ventricular End-diastolic Diameter (cm)		6.3±0.3
Left Ventricular End-systolic Diameter (cm)		5.7±0.3
Fractional Shortening (%)		13±0.2
Left Ventricular Ejection Fraction		19±2%
Concomitant Therapy		
β-blockade		10/12
Angiotensin-Converting Enzyme Inhibitor/Angiotensin Receptor Blocker	1	11/12
Aldosterone Receptor Antagonist		5/12
Implanted Cardio-defibrillator		5/12
Digoxin		3/12

Aspirin	11/12
Statin	6/12
Loop Diuretic Therapy	4/12
Warfarin	3/12

TABLE 6.3 Baseline haemodynamic data

	(Pyr[1])apelin-13	Placebo
Cardiac Index L/min/m^2	2.9±0.2	3.0±.03
Heart Rate beats/min	61.3±4.2	58.1±3.0
Systolic Blood Pressure mmHg	128.2±7.4	126.2±6.3
Diastolic Blood Pressure mmHg	75.5±3.9	72.1±2.8
Mean Arterial Pressure mmHg	93.4±4.1	85.5±4.3
Peripheral Vascular Resistance Index dynes.s/cm^5/m^2	2718±236	2457±339
Left Ventricular End-diastolic Diameter (cm)	6.3±0.3	6.3±0.3
Left Ventricular End-systolic Diameter (cm)	5.8±0.3	5.6±0.3
Fractional Shortening (%)	10±1	13±1
Left Ventricular Ejection Fraction (%)	21±3	25±2

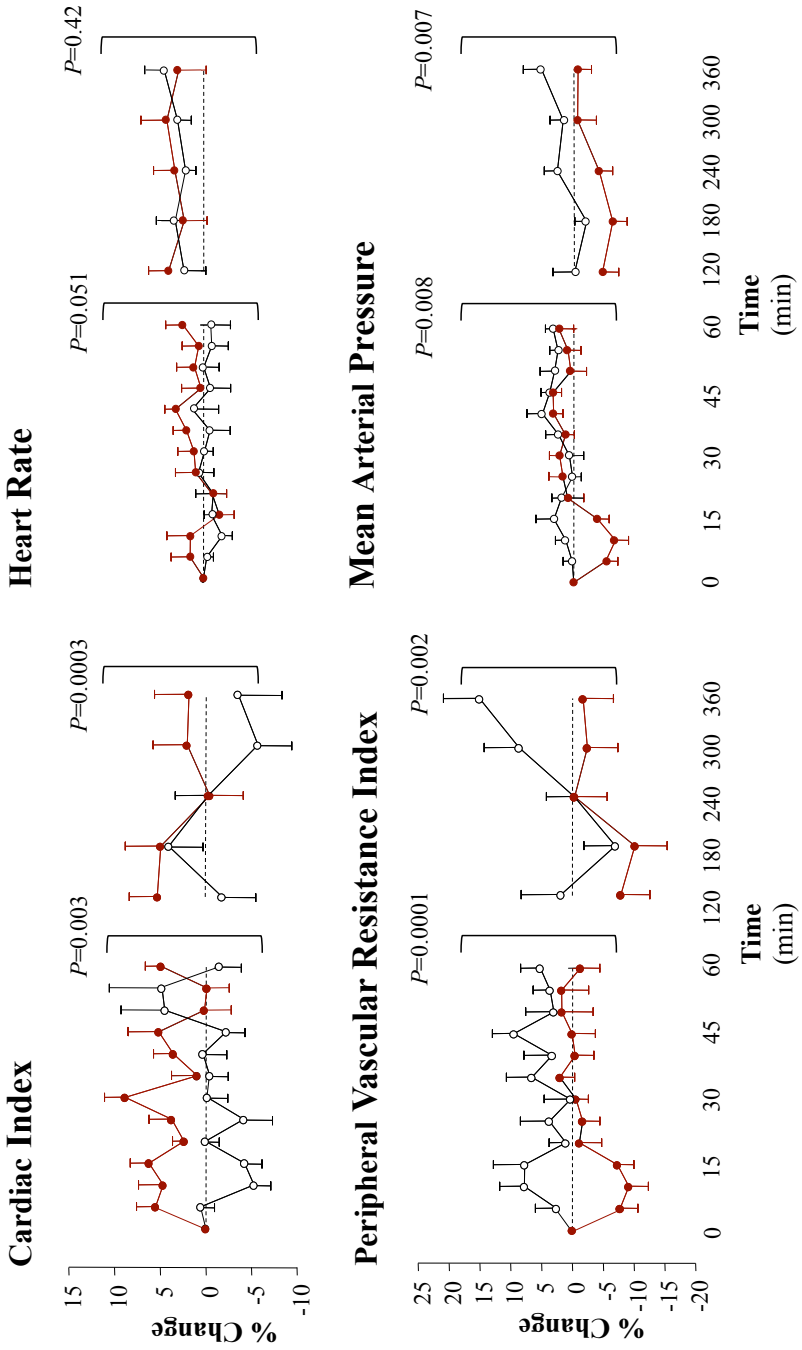

Figure 6.3. Percentage change in cardiac index, mean arterial pressure, peripheral vascular resistance index and heart rate during systemic infusion of (Pyr[1])apelin-13 (red circles, closed) or saline (black circles, open). **P* <0.05, ***P* <0.01, ****P* <0.001. One-way ANOVA with a post-hoc Bonferroni test. ANOVA - analysis of variance.

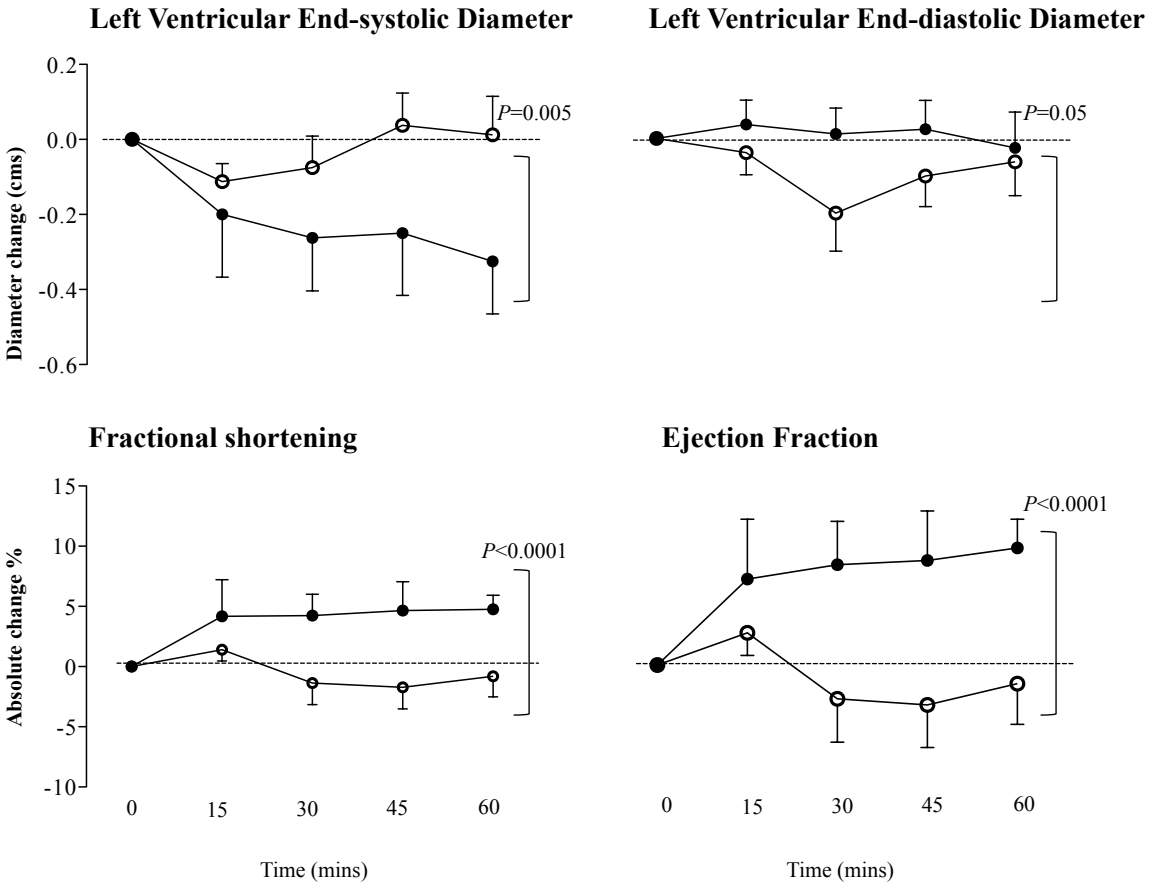

Figure 6.4. Absolute change in left ventricular end-systolic (cm), end-diastolic measurements (cm), fractional shortening and ejection fraction (absolute percentage change) during systemic infusion of (Pyr[1])apelin-13 (black circles, closed) or saline (black circles open). **P* <0.05, ***P* <0.01, ****P* <0.001. Two-way ANOVA with a post-hoc Bonferroni test.
ANOVA - analysis of variance.

6.5 DISCUSSION

This is the first study to assess prolonged systemic (Pyr^{1})apelin-13 infusion in man. In both healthy volunteers and patients with chronic stable heart failure, the effects on the cardiac index were sustained during infusion. No serious adverse events were observed during this study.

6.5.1 SAFETY

Throughout all the studies, (Pyr^{1})apelin-13 infusion was well tolerated. Two studies were stopped prematurely in patients with heart failure: one due to an episode of long-standing chronic backache and the other one due to uncontrolled hypertension, which was pre-existing and controlled with medical therapy. It is important to note that no cardiovascular symptoms were reported in either of these studies. Additionally, no arrhythmias were recorded. Whilst no clinical studies have identified (Pyr^{1})apelin-13 to be proarrhythmic [Japp *et al* 2008; Japp *et al* 2010], one preclinical study recorded various degrees of atrioventricular node blockage in response to apelin infusion [Charles *et al* 2006]. Reassuringly, (Pyr^{1})apelin-13 infusion throughout this study did not provoke arrhythmias in healthy volunteers or in patients with chronic stable heart failure. The present study provides valuable information regarding the safety profile of APLNR agonism, although chronic dosing may have a very different safety profile.

6.5.2 EFFICACY

In both healthy volunteers and patients with chronic stable heart failure, systemic (Pyr[1])apelin-13 infusion is safe and results in sustained increased cardiac output, with no evidence of tachyphylaxis. The magnitude of effect of (Pyr[1])apelin-13 infusion in this trial is similar to previously reported studies [Japp *et al* 2010]. In patients with chronic stable heart failure we report improvements in left ventricular dimensions, which is in keeping with increased cardiac output.

A major concern of targeting the APLNR is the potential for tachyphylaxis: a reducing response to continued dosing. In man, the local vascular and systemic cardiovascular responses to APLNR agonism demonstrate a rapid plateau in the dose response curve, as presented in this thesis and in keeping with previously published data [Japp *et al* 2008; Japp *et al* 2010]. This may represent tachyphylaxis and have serious implications for the therapeutic use of (Pyr[1])apelin-13. Furthermore, concerns exist regarding the rapid internalisation of the APLNR [Messari *et al* 2004], which localises to a perinuclear cellular location once activated. Whilst preclinical data are limited, ventricular APLNR expression is reduced in dilated cardiomyopathy, and this may reduce the capacity of APLNR signalling for this type of heart failure [Földes *et al* 2003; Pitkin *et al* 2010]. However, varying results are reported in patients with heart failure secondary to the ischaemic heart disease, with both reduced [Pitkin *et al* 2010] and preserved ventricular expression patterns reported [Földes *et al* 2003]. From the data in the present study the effect of systemic (Pyr[1])apelin-13 was not reduced in chronic stable heart failure, suggesting that there is sufficient receptor density to target. The majority of patients recruited to this study had ischaemic heart failure, which may be an important factor in

the response, although patients with dilated cardiomyopathy had a similar profile of response.

The exact mechanism underlying the increased cardiac index is difficult to ascertain from this study. In preclinical studies, using a range of preclinical models from isolated myocytes to whole animal studies [Berry *et al* 2004; Dai *et al* 2006; Farkasfalvi *et al* 2007], apelin has been demonstrated to be a potent positive inotrope. In keeping with this, invasive haemodynamic preclinical studies have shown a direct effect on the myocardium [Ashley *et al* 2005]. Nevertheless, as evidenced in peripheral arterial studies and systemic infusions, apelin functions as a vasodilator. In both protocols a reduction in afterload was evident throughout the first hour of (Pyr^{1})apelin-13 infusion. The response differed between healthy volunteers and patients with chronic stable heart failure, with the healthy cohort showing an increase in the cardiac index, without a sustained reduction in mean arterial pressure. In patients with chronic stable heart failure, (Pyr^{1})apelin-13 infusion resulted in reduced afterload compared to the placebo throughout the duration of infusion. It is likely that reduced afterload contributes to increased cardiac output, but the reduction in afterload was modest relative to the increase in the cardiac index, thus suggesting that there is a direct effect on the myocardium.

We have studied two different populations in this protocol. The healthy volunteers were younger (21.1±0 yrs) and all male, compared to the chronic heart failure cohort (mean age 64±3 yrs), with both males and females studied. We have seen similar cardiovascular responses on each group, although no tachycardia was recorded in the patient group, in

either the first hour or during the 6-hour analysis. This is likely to reflect concurrent treatment with β-blockers and digoxin.

We have addressed potential concerns surrounding APLNR signalling in heart failure and failed to demonstrate evidence of tachyphylaxis over the timescale investigated. The duration of infusion in these studies was 6 hours, compared to previous studies in humans that have been limited to 15-minute infusions [Japp *et al* 2008; Japp *et al* 2010]. Nonetheless, this still reflects a relatively short infusion, and if the APLNR is to develop as a therapeutic target in chronic heart failure, data are required to assess a longer APLNR agonism duration. Whilst no arrhythmias were recorded in these studies, careful consideration and monitoring would be necessary should the APLNR be a target in heart failure. Studies of inotropes in heart failure have reported hazardous effects, and it is conceivable that chronic stimulation of the myocardium will be proarrhythmic and harmful [Miller *et al* 2008].

Beyond the haemodynamic benefits that we have demonstrated, some chronic effects may also be beneficial. In the earlier chapters we established that there is no haemodynamic interaction between the apelin-APLNR and renin-angiotensin system, although preclinical data have shown that these two systems do in fact interact. In preclinical models, apelin inhibits angiotensin II-mediated cardiac fibrosis [Siddiquee *et al* 2011], and this property would be very attractive in the treatment of ventricular dysfunction. Investigating any beneficial effects of APLNR agonism beyond haemodynamic properties, such as ventricular remodelling seen in patients treated with

ACE inhibitors [Konstam *et al* 1992] or β-blocker therapies [Doughty *et al* 2004], would require chronic dosing.

All of the patients investigated were receiving current evidence-based therapies in heart failure, with over 90% receiving some form of renin-angiotensin inhibition. The contribution of these agents to APLNR agonism would be interesting to study. However, it would not be ethical to withhold treatment of medications that improve mortality, in order to assess their contribution to (Pyr^1)apelin-13 efficacy. Furthermore, this may result in decompensating their heart failure, which again would not be ethical.

Whilst an oral preparation may not be available at present, the duration of infusion in this study may be suitable for the treatment of acute heart failure. Nevertheless, in recent times many drugs have had favourable haemodynamic and neuroendocrine profiles [Colucci *et al* 2000; Publication Committee for the VMAC Investigators 2002; Silver *et al* 2002; Gheorghiade *et al* 2004; Konstam *et al* 2007], although these have not been associated with improved outcomes. Acute heart failure represents a heterogeneous patient group with regard to the aetiology of ventricular dysfunction and decompensation, with a long, maladaptive and compensatory response duration. A brief infusion of any drug is therefore unlikely to improve long term outcomes.

6.5.3 LIMITATIONS

The healthy volunteer population is not representative of the population at large; the cohort investigated in these studies was young and exclusively male. Extrapolating these findings to the wider population may not be relevant. Nonetheless, the heart failure

population investigated is representative of a typical patient population with respect to aetiology, demographics and current therapy.

In this present study we used thoracic electrical bioimpedance to assess cardiac output, and echocardiography to assess left ventricular dimensions. Whilst good correlation exists between invasive techniques [Shoemaker *et al* 1994; Suttner *et al* 2006; Gujjar *et al* 2008; McDonagh *et al* 2009], the level of agreement is not always acceptable in all studies [Leslie *et al* 2004]. The optimal technique employed to ascertain myocardial effects would be to undertake pressure-volume loop studies that give information regarding cardiac function independent of loading. However, it is ethically challenging to use invasive techniques to assess cardiac output over a 6-hour period, and it is not routinely or readily available in many centres.

Whilst echocardiography data are in concordant with the haemodynamic effects seen with thoracic impedance cardiography assessment of the left ventricle was limit to one plane and not possible in all individuals studied. In order to preserve thoracic impedance cardiography traces, patients we requested to remain still throughout the measuring period and therefore assessment from any other axis was not possible. Moreover, there is no reliable technique to ensure measurements are obtained from the same position of the myocardial wall during each sample.

Given that values obtained for baseline fractional shortening are low in heart failure cohort recruited, to small changes in left ventricular dimensions will be sensitive to small changes in dimension. This effect will be amplified when using the Teichholz

method, which cubes left ventricular dimensions, and makes assumptions regarding left ventricular geometry. Teichholtz method has been reported to overestimate ejection fraction, with a higher coefficient a variability in heart disease compared to healthy individuals (14.8% vs 5.1%) [Wandt *et al* 1999] Alternative echocardiographic methods could have been used to assess ejection fraction, such as Simpson's biplane method, to produce a more precise assessment of ejection fraction. The use of echocardiogram contrast agents would have improved endocardial definition and may have permitted inclusion of more patients. Alternatively, other modalities such as magnetic resonance imaging would provide more accurate assessments of intracardiac volumes [Bellenger *e*t al 2000]. Overall echocardiographic data support the findings presented within this study, however should be interpreted with caution given the know limitations of the techniques used, and serve to guide further investigation if the effect on APLNR stimulation on ejection fraction is to be determined.

At present there is no acceptable method to measure plasma apelin concentration and none of the commercially available assays is able to detect (Pyr^{1})apelin-13, which is the most potent fragment and is abundant in cardiac tissue, and therefore the peptide of most interest. We have consequently been unable to assess plasma concentrations as a result of (Pyr^{1})apelin-13 infusion in this study, which would have been helpful in understanding the magnitude of effect.

6.5.4 SUMMARY

This protocol provides essential data to guide future work in assessing the role of exogenous apelin in man. There are limited data in this respect, with infusions restricted to around 15 minutes, but this protocol provides novel information regarding the more prolonged cardiovascular effects of apelin in man. It is encouraging to note that the effects persist in patients with heart failure and are not restricted solely to healthy volunteers. We predict that APLNR agonism will retain efficacy and is a valid pharmacological target in heart failure.

CHAPTER 7

INVESTIGATING THE EFFECT OF EXOGENOUS (PYR[1])APELIN-13 ON EXERCISE PERFORMANCE

7.1 SUMMARY

Introduction In preclinical studies, rodents deficient in apelin have reduced exercise tolerance suggesting an important role for the apelin-APLNR system in exercise physiology.

There are no clinical data assessing either the effect of apelin on exercise or the dynamic response of plasma apelin during exercise. The aim of this study was therefore to assess the effect of exogenous apelin infusion during exercise and the effect of exercise on plasma apelin concentration.

Methods Twelve healthy volunteers attended three cardiopulmonary exercise tests. On the first visit, an incremental protocol was undertaken to ascertain VO_{2MAX}, and endurance protocols were performed on subsequent visits during (Pyr^{1})apelin-13 30 nanomol/min or saline infusion, administered in a double blinded randomised crossover design. Electrocardiograph recordings, endurance time, oxygen pulse heart rate and blood pressure were recorded throughout with an ergometer, metabolic cart and semi-automated sphygmomanometer respectively. Blood samples were drawn at baseline and during peak exercise.

Results No adverse events were recorded during this study. Exogenous (Pyr^{1})apelin-13 infusion had no effect on endurance performance. However, there was an increase in plasma apelin concentration in response to exercise.

Conclusion This is the first study in man to assess the (Pyr^{1})apelin-13 infusion during exercise. Exogenous apelin infusion had no effect on endurance times, however an increase in plasma apelin concentration was observed. These data suggest endogenous APLNR activation does not improved exercise performance in healthy individuals. Assessing the effect of exogenous apelin in patients with heart failure, who have reductions in plasma apelin, merits investigation as this cohort may have insufficient APLNR activation during exertion.

7.2 INTRODUCTION

The apelin-APLNR system is implicated in having a significant role in cardiovascular regulation. Its primary actions in the circulatory system are to mediate positive inotropism [Szokodi *et al* 2002; Berry *et al* 2004; Farkasfalvi *et al* 2007; Japp *et al* 2010] and vasodilatation [Lee *et al* 2000; Japp *et al* 2008; Japp *et al* 2010].

Apelin is the most potent inotrope identified and is effective at subnanomolar concentrations [Szokodi *et al* 2002], as investigated in many preclinical models, ranging from isolated myocytes to whole animal models. In humans, exogenous apelin has been shown to increase cardiac output in both healthy volunteers and patients with chronic stable heart failure [Japp *et al* 2010].

Whilst the inotropic action of the apelin-APLNR system has been well described under resting conditions, there are limited data on the contribution of this system to

exercise performance. Studies assessing the contribution of the apelin-APLNR system during exercise are limited to knockout models. In rodent models deficient in either APLNR or apelin, deficiencies in exercise performance are observed. VO_{2MAX} is reduced in apelin-deficient mice [Charo *et al* 2009], suggesting that the apelin-APLNR system contributes to ventricular performance in exercise.

Plasma apelin concentrations have been reported in healthy individuals and a range of diseases. There is general agreement that plasma apelin concentrations are reduced in heart failure [Chen *et al* 2003; Földes *et al* 2003; Chong *et al* 2006; Francia *et al* 2007] and a causal role is implied. In less severe heart failure an increase in plasma is reported, signifying an initial adaptive attempt to augment myocardial contraction, which is greatly reduced in severe heart failure [Chen *et al* 2003]. Studies in man profiling apelin-APLNR expression responses, in response to ventricular or biventricular pacemaker insertion, show increases in apelin-APLNR as ventricular function improves [Chen *et al* 2003; Francia *et al* 2007]. It is conceivable that exogenous apelin given to these patients groups may translate into a functional benefit. There are no data profiling plasma apelin in response to exercise or the effects of exogenous apelin during exercise.

The aims of this protocol were to assess the safety and efficacy of exogenous apelin during exercise, primarily to investigate the ergogenic effect of apelin infusion and to assess circulating levels of apelin peptide during exercise.

7.2.1 HYPOTHESIS

Exogenous (Pyr[1])apelin-13 will increase endurance time and plasma apelin concentrations will increase during exertion.

7.3 METHODS

Twelve healthy volunteers attended on three occasions, with each visit separated by one week, for cardiopulmonary exercise testing. One dataset was withdrawn prior to unblinding, due to submaximal exertion attributed to a chest infection that was not disclosed prior to the study visit. All studies were performed in a quiet, temperature controlled room maintained at 22-24°C. On the first visit, the subjects performed a ramp incremental symptom-limited endurance protocol, in order to ascertain maximal workload. Subjects initially sat resting on the ergometer for 2 minutes and thereafter pedalled for 1 minute with no load applied. Workload was then increased incrementally and the test continued until symptoms limited further effort. Encouragement was given throughout the tests to maximise the subjects' exertion. From visit, the maximal work-load each participant was capable of was recorded. Subsequently, participants attended visits for two further visits that were endurance protocols (Figure 7.1), in which the workload was set at 80% of the maximal, as previously reported [Greer *et al* 2000; Oga *et al* 2003; Vagaggini *et al* 2011]. 2 minutes of rest were followed by 1 minute of unloaded cycling prior to the endurance exercise tests. Breath-by-breath analysis was recorded throughout, and data were collected automatically.

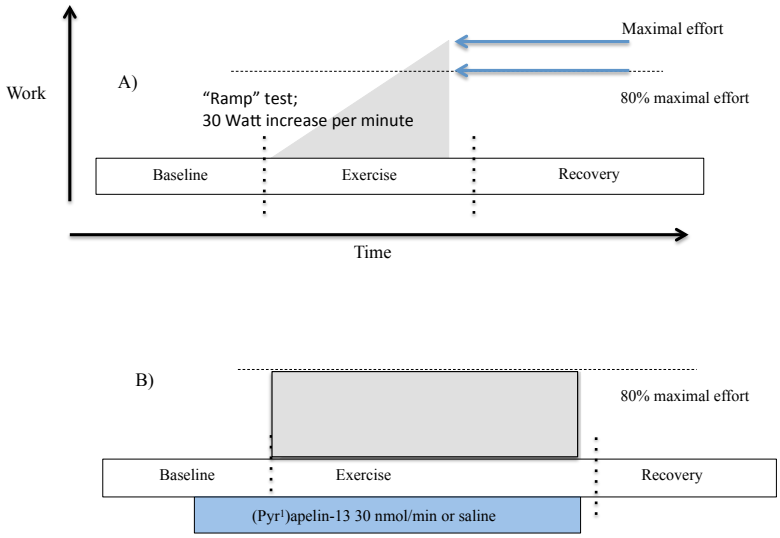

Figure 7.1 Study protocol. **A)** Visit 1: Maximal effort incremental cardiopulmonary exercise test. **B) Visit 2 and 3**: Endurance cardiopulmonary exercise test at 80% maximal workload.

In a crossover design subjects were randomised in a double blinded fashion to receive a (Pyr^{1})apelin-13 (30 nmol/min) (Clinalfa AG, Läufelfingen, Switzerland) infusion or a matched saline placebo infusion through a peripheral cannula at 1 mL/min during endurance exercise testing. Blood pressure, heart rate, gas exchange and electrocardiogram were recorded throughout the protocol. Blood samples were drawn at baseline and at peak exercise and recovery from the non-infused arm during endurance exercise testing. Prior to undertaking exercise testing, spirometry was performed.

7.3.1 PLASMA APELIN

Plasma apelin was assayed using standard, commercially available ELISA assay and processed in accordance with the manufacturer's instructions. Briefly, bloods were drawn into EDTA vacutainers, centrifuged at 4^{o}C, 1500 rpm for 20 minutes and stored at -80^{o}C. A protein extraction phase was undertaken (C18 extraction column, Phoenix Peptides) and ELISA performed thereafter. Plasma apelin concentrations were assessed during the placebo visit, as the current assay does not detect the pyroglutamated form of apelin-13.

7.3.2 DATA AND STATISTICAL ANALYSIS

Endurance time, mean arterial pressure, oxygen pulse, ventilation efficiency and heart rate during (Pyr^{1})apelin-13 infusion were compared with those during the

placebo infusion. Plasma apelin concentration at baseline and peak exercise was assessed from the placebo visit. All were analysed by paired two-tailed Student's *t*-test using GraphPad Prism. Statistical significance was taken as $P < 0.05$. Based on previous studies investigating cardiopulmonary exercise testing we calculated that to see a 20% effect on exercise performance we will need a sample size of 12 at 90% power and two side $p<0.05$.

7.4 RESULTS

7.4.1 INCREMENTAL EXERCISE TESTING

APLNR agonism was assess with (Pyr^{1})apelin-13 (30 nmol/min) (Clinalfa). Baseline characteristics and workloads during incremental testing are presented in Table 7.1. Subjects were young males with moderate exercise ability. One data set was withdrawn prior to analysis as the participant had coryzal symptoms on the last visit, we therefore present data for 11 participants. Incremental exercise test effort was satisfactory, with respiratory exchange ratio >1.0 accepted as supramaximal effort.

7.4.2 SAFETY

All studies were well tolerated, with no adverse events. In particular, no cardiovascular symptoms were reported during either endurance exercise test, and no electrocardiogram abnormalities were evident during (Pyr^{1})apelin-13 infusion (Figure 7.2).

TABLE 7.1 Baseline characteristics of the participants

Baseline Characteristics	
Age (yrs)	29±3
Weight (kg)	78±3
Height (m)	1.8±0.0
BMI (kg/m^2)	24.6±0.5
Forced Expiratory Volume (L)	5.0±0.3
Forced Vital Capacity (L)	6.3±0.3
VO_{2MAX} (mL/min/kg)	46.0±3.0
Incremental Work (W)	303.0±24.5
Respiratory Exchange Ratio	1.2±0.0

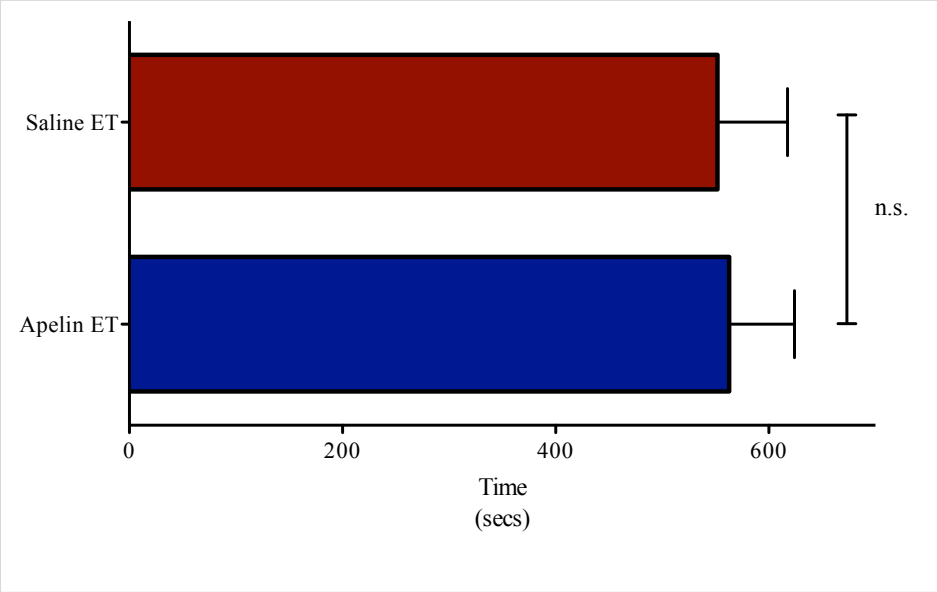

Figure 7.2. Endurance time during (Pyr[1])apelin-13 infusion (blue) or saline (red). Paired Student's *t*-test (*P*=ns).
ET - endurance time; ns - not significant**.**

7.4.3 EFFICACY

Intravenous (Pyr[1])apelin-13 was not associated with increased endurance time (563s *versus* 552s; paired Student's *t*-test, *P*=ns). No differences in heart rate or mean arterial pressure were detected throughout endurance testing, however oxygen pulse was reduced during (Pyr[1])apelin-13 infusion (paired Student's *t*-test, *P*=ns). (Pyr[1])apelin-13 infusion reduced oxygen pulse during exercise (23.1±1.2 mL/kg *versus* 21.7±1.1 mL/kg; paired Student's *t*-test, *P*=0.0015), while the respiratory exchange ratio was similar during each endurance test and the values obtained throughout are consistent with supramaximal effort (Table 7.2).

TABLE 7.2 Haemodynamic parameters at peak exercise during placebo or (Pyr[1])apelin-13 visits

	Placebo	(Pyr[1])apelin-13
Respiratory Exchange Ratio	1.1±0.0	1.1±0.0
Oxygen Pulse (mL/beat)	23.1±1.3	21.7±1.1*
Heart Rate at Maximal Exertion (bpm)	170±5	174±4
Mean Arterial Pressure at Maximal Exertion (mmHg)	111±4	117±4
Endurance Time (s)	551.8±65.9	562.8±61.4

Paired Student's *t*-test. **P* <0.05, ***P* <0.01, ****P* <*0.001*.

7.4.4 PLASMA APELIN CONCENTRATION

Paired baseline and exercise sample were available for 8 participants. Plasma apelin concentrations were increased in response to exercise. Baseline plasma apelin concentrations were 0.17±0.06 ng/mL rising to 0.26±0.1 ng/mL at peak exercise (*P*=0.05) (Figure 7.3).

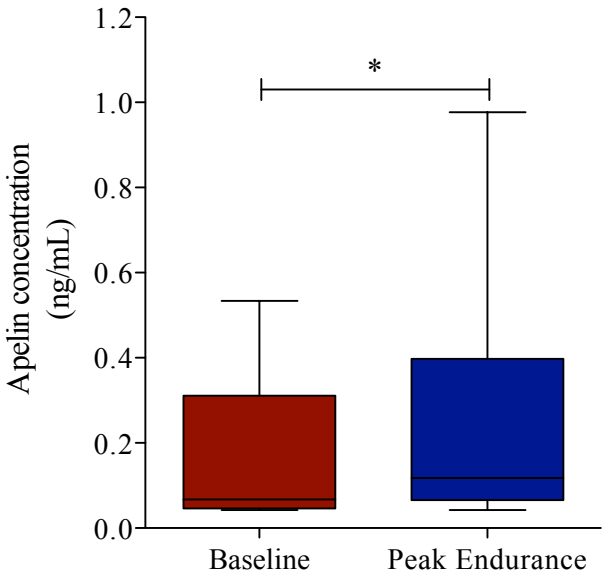

Figure 7.3. Baseline plasma apelin concentration (red), peak exercise apelin concentration (blue). **P* <0.05, ***P* <0.01, ****P* <0.001. Paired Student's *t*-test.

7.5 DISCUSSION

This is the first study in man to assess the effects of exogenous (Pyr^{1})apelin-13 infusion during exercise. We can report that no effect on endurance time, heart rate or mean arterial pressure during exercise was observed in this study.

Under rest conditions, APLNR agonism consistently mediates positive inotropic effects, observed in both preclinical and clinical studies, in both healthy and failing myocardium. However, there is a paucity of data assessing the contribution of the apelin-APLNR system to exercise. Animal models, deficient in either apelin or APLNR, have been shown to have reductions in basal cardiac contractile indices [Charo *et al* 2009] that functionally translate to a reduced exercise capacity. Furthermore, apelin-deficient rodents rapidly develop heart failure in response to an increased afterload, which is in keeping with a requirement of this system to function under conditions of stress [Kuba *et al* 200]), although this may reflect a chronic rather than an acute effect. In this study, we have failed to demonstrate any effect of exogenous apelin in healthy individuals exercising at high workloads, suggesting that under these conditions exogenous APLNR stimulation is not ergogenic.

The (Pyr^{1})apelin-13 dose infused in this study was in line with other clinical studies [Japp 2010] and sufficient to activate the APLNR; therefore, we do not believe that any lack of effect results can be attributed to inadequate dosing. These concentrations have

been shown as effective doses in both the acute and subacute settings in increasing cardiac output and reducing mean arterial pressure.

Endurance protocols set at 80% of VO_{2MAX}, with similar dosing protocols, have been used in both healthy volunteers [Graham and Spriet 1995; Greer *et al* 2000] and patient groups [O'Donnell and Lam 1998; Oga *et al* 2003; O'Donnell 2006; Salcedo *et al* 2007; Guenette *et al* 2011; Vagaggini *et al* 2011] and been able to detect changes in endurance times. In trained athletes caffeine supplementation prior to exercise extends endurance time, while in chronic obstructive airways disease, acute bronchodilator therapy increased endurance time by around half a minute. We do not believe the lack of effect was therefore related to selecting an inappropriate workload, as adequate stress levels were selected in this protocol. Furthermore, these studies employed acute dosing techniques, similar to those in this study, and were able to detect treatment effects. Thus, in populations that were trained more and trained less, a similar study design has been able to detect changes in endurance. However there may have been subtle effects of APLNR agonism on exercise capacity this study was not powered adequately to detect.

APLNR activation has no clear dose response relationship, with the effects plateauing rapidly [Japp *et al* 2008; Japp *et al* 2010]. It is possible that exercise releases endogenous stores of apelin that saturate the available APLNR, resulting in no additional exogenous apelin effect. We report results from a healthy cohort, and it is conceivable that in disease populations the effects of exogenous apelin may differ.

There may have been off-target effects from exogenous apelin infusion. Oxygen pulse, determined by stroke volume, reduced during (Pyr^1)apelin-13 infusion and exercise. No difference in heart rate or afterload was noted between the two endurance visits, which would suggest that the observed difference was explained by either contractility or preload. Given that all studies investigating the effect of the apelin-APLNR system on myocardial contractility reported positive inotrope effects, a reduction in preload may be the most plausible explanation. This may be explained by vasodilatation in vascular beds that would normally be constricted. No assessment of blood flow through vascular beds was made during this protocol, but it is possible that APLNR agonism mediated atypical volumes in redistribution during exercise.

An increase in plasma apelin was detected, suggesting that this endogenous hormone may have a role in healthy exercise physiology. Alternatively, exercise may be a stimulus for apelin production, rather than actively increasing cardiac output. However, given data from preclinical studies, and the data presented in this thesis, it is reasonable to propose a role for apelin in exercise physiology. There are concerns regarding the assay that is currently available, and there is no cross-reactivity with pyroglutamated apelin. Considering that (Pyr^1)apelin-13 is the most abundant isoform of apelin in cardiac tissue and is recognised to be the most potent isoform, understanding its contribution to exercise is essential. This cohort is a healthy well-trained population, so whether or not these dynamic changes are similar in disease remains to be ascertained.

7.5.1 LIMITATIONS

This is the first study infusing apelin during exercise and has correctly been performed in a small number of healthy volunteers. These data may not be informative with respect to patient studies.

We tested endurance at one level, which is in keeping with reported literature [Graham and Spriet 1995; Greer *et al* 2000]. However it may have been more informative to test individuals over a range on endurance workloads and assess the effect of exogenous apelin at even more extreme workload. This would have resulted in increasing the number of visits and may have introduced both training and learning effects.

We present data showing a rise in plasma apelin during exercise. However, there are concerns regarding the accuracy of this assay and caution is required. Furthermore we were only able to analyse a limited number of paired samples owing to difficulty in sampling some individuals during exercise. Given that the cardiac atria are suggested to be a major source of circulating apelin, arterial samples may be more informative regarding plasma apelin concentration during exercise, we have collected venous samples as this negates the need for invasive arterial cannulation.

There are no APLNR antagonists available at present, however this would be beneficial in understanding the role of the apelin-APLNR system in exercise. We

propose that endogenous APLNR contributes to exercise, but cannot be augmented with exogenous (Pyr^{1})apelin-13, treatment with and antagonist would result in a reduction in exercise performance.

7.5.2 CONCLUSIONS

Exogenous (Pyr^{1})apelin-13 infusion during cardiopulmonary exercise testing progressed with no adverse effects, and in particular we did not detect any arrhythmias. Exogenous (Pyr^{1})apelin-13 had no effect on exercise duration in this study. However, this study protocol was restricted to healthy volunteers and it is conceivable that different effects may be observed in diseased populations.

CHAPTER 8

CONCLUSIONS AND FUTURE DIRECTIONS

8.1 SUMMARY OF THE FINDINGS

The role of the apelin-APLNR system in cardiovascular health and disease is becoming increasingly better defined. Apelin receptor activation mediates arterial and venous vasodilatation which is predominantly endothelial and nitric oxide-dependent [Lee *et al* 2000; Tatemoto *et al* 2001; Ishida *et al* 2004; Japp *et al* 2008], whilst it is also reported as the most potent inotrope *in vitro* [Szokodi *et al* 2002]. In a range of preclinical and clinical studies this effect has been consistently demonstrated, and *in vivo* in humans this effect persists, albeit with reduced potency [Japp *et al* 2010].

The APLNR is a G protein-coupled receptor that is most closely related to the AT1R [O'Dowd *et al* 1993]. Each of these receptors is located in similar locations throughout the body, but their cardiovascular actions are largely opposed [Ashley *et al* 2006]. In addition to opposing actions on vascular tone [Gurzu *et al* 2006], inflammation [Chun *et al* 2008] and fluid balance [Hus-Citharel *et al* 2008], there appears to be a direct interaction between the APLNR and AT1R. The inhibition of AT1R expression results in an increase in APLNR expression and a reduction in mean arterial pressure in a hypertensive rodent model, whilst elevated angiotensin II reduces cardiac apelin expression [Iwanaga *et al* 2006]. Furthermore, preclinical data suggest that there is a direct interaction between each of these receptors and they are able to form heterodimers, which in turn impacts on intracellular signalling [Chun *et al* 2008; Sun *et al* 2011]. APLNR stimulation inhibits renin-angiotensin signalling

and has been shown in preclinical studies to reduce angiotensin II cardiac fibrosis [Siddiquee *et al* 2011].

Renin-angiotensin activation is deleterious to health in a range of diseases, but this is particularly true of cardiovascular disease and heart failure. Pharmacological inhibition results in reduced mortality [Cleland *et al* 1997; Swedberg *et al* 1999], and importantly, added survival benefits are seen in patients treated with more than one class of renin-angiotensin medication [McKelvie *et al* 1999; Pfeffer *et al* 2003; Young *et al* 2004].

The potential of the apelin-APLNR system may be 2-fold; firstly it can improve myocardial performance and secondly it may antagonise the renin-angiotensin system.

8.1.1 SUBACUTE RENIN-ANGIOTENSIN DOES NOT ALTER THE LOCAL OR SYSTEMIC CARDIOVASCULAR ACTIONS OF (PYR^{1})APELIN-13

In a randomised single blinded crossover study, 12 healthy volunteers were assigned to a salt-restricted (<12 mmol Na^{+} with a single dose of furosemide) or a normal diet. In this study there was good adherence to the sodium deplete diet and significant increases in renin-angiotensin system activation were achieved. The local and systemic cardiovascular actions of (Pyr^{1})apelin 13 were unaffected by subacute renin-angiotensin activation. We predict that (Pyr^{1})apelin 13 will retain efficacy in the presence of prolonged renin-angiotensin activation.

8.1.2 ACUTE ANGIOTENSIN II DOES NOT ALTER THE LOCAL SYSTEMIC CARDIOVASCULAR ACTIONS OF (PYR^{1})APELIN-13

Thereafter, in order to assess any immediate interaction between the apelin-APLNR and renin-angiotensin systems, we performed a randomised double blinded crossover study, recruiting 12 healthy volunteers, and administered angiotensin II or placebo infusion. Local intrabrachial arterial angiotensin II infusion was titrated to a reduced forearm blood flow by 50%, and in the systemic studies a subpressor angiotensin II dose was administered. In both local and systemic acute angiotensin II infusion the cardiovascular actions of apelin persisted. Preclinical data point to the formation of heterodimers between the APLNR and AT1R which alters signal transduction from their respective ligands. In this study we have not been able to demonstrate any acute interaction between the apelin-APLNR and renin-angiotensin systems.

8.1.3 INVESTIGATING THE CARDIOVASCULAR EFFECTS OF (PYR^{1})APELIN-13 IN CHRONIC STABLE HEART FAILURE

Given the downregulation of myocardial APLNR expression and the reduction in circulating plasma apelin, in heart failure, the effect of exogenous apelin infusion was assessed. Whilst brief, the concerns regarding safety and efficacy were addressed during this study. Eight patients with chronic stable heart failure were investigated in a randomised double blind crossover design. Systemic infusion of (Pyr)apelin-13 increased cardiac output and there was a concomitant reduction in afterload. During these studies no serious adverse events were reported, and it is noteworthy that no arrhythmias were recorded. However, a rapid plateau was evident in response to ascending doses, thus suggesting tachyphylaxis. Additionally, this

may also represent reduced myocardial APLNR density and a system that is readily exhausted.

8.1.4 INVESTIGATING THE CARDIOVASCULAR EFFECTS OF PROLONGED (PYR[1])APELIN-13 INFUSION IN HEALTHY VOLUNTEERS AND PATIENTS WITH CHRONIC STABLE HEART FAILURE

Given concerns over tachyphylaxis and the potential reduction of APLNR density, it was necessary to assess the cardiovascular response to (Pyr[1])apelin-13 infusion over a prolonged period. In these studies we extended the duration of apelin infusion in humans 25-fold, therefore representing the longest duration of exogenous apelin infusion *in vivo* in humans. A randomised double blinded placebo control crossover study was performed in healthy volunteers and patients with chronic stable heart failure, with 12 participants recruited into each study. All of the infusions were well tolerated and no serious adverse events were recorded. As with the brief infusions, but of more importance given the increase in duration, no arrhythmias were captured. During the 6-hour infusion the effects of (Pyr[1])apelin-13 persisted and showed no evidence of tachyphylaxis. Similar increases in the cardiac index were observed in both the healthy controls and the patient cohort. Throughout the initial hour of infusion, left ventricular dimensions were recorded in the patient group and improved left ventricular performance was evident. This study demonstrated that APLNR signalling persists over a prolonged infusion period, and importantly the effect persists in patients with chronic stable heart failure.

8.1.5 INVESTIGATING THE EFFECT OF (PYR[1])APELIN-13 ON EXERCISE PERFORMANCE

In this study we explore the effect of exogenous (Pyr[1])apelin-13 infusion during exercise. Exogenous (Pyr[1])apelin-13 in man is inotropic when infused under resting conditions and during acute and prolonged infusion, in both healthy volunteers and patients with chronic stable heart failure. No study has previously assessed the effect of APLNR stimulation during exercise. Given the proposed role in ventricular function, this important study addressed the contribution of exogenous (Pyr[1])apelin-13 infusion during exercise in healthy volunteers. Eleven healthy volunteers performed an endurance cardiopulmonary exercise test in a random double blinded crossover design, following the determination of VO_{2MAX} on incremental testing. Exogenous apelin had no effect on endurance times in this cohort. In healthy volunteers it is possible that there is no, or minimal, contribution of apelin or that endogenous activity cannot be supplemented. This merits further investigation in patients with ventricular failure, in order to assess the effect of targeting the APLNR during exercise.

8.2 FUTURE DIRECTIONS

8.2.1 CARDIOVASCULAR

In this thesis we have presented data assessing the efficacy of the APLNR agonist in the context of renin-angiotensin upregulation and profiled the cardiovascular response to acute and prolonged apelin infusion in chronic stable heart failure. We conclude that apelin functions independently of angiotensin II, and the APLNR is a

potentially exciting therapeutic target. At present, the main barriers to exploring the apelin-APLNR system in heart failure are the lack of an oral compound and the fact that it is challenging to use prolonged infusion. Preclinical data support an antifibrotic role in myocardium, and in particular it abrogates angiotensin II-induced fibrosis [Siddiquee *et al* 2011]. Clearly, any beneficial effect on myocardial remodelling would be of great value, provided that there was an accompanied improvement in ventricular function that translated to a reduction in both morbidity and mortality. At present, there is little scope to assess these parameters in patients with chronic stable heart failure.

Whilst we were unable to identify any interaction between the apelin-APLNR and renin-angiotensin systems in the studies performed, the protocols were limited to brief studies. That we did not see an interaction does not exclude the possibility that an interaction exists and APLNR agonism may inhibit the renin angiotensin system. In the longer term, using chronic dosing regimens, APLNR agonsim may be synergistic with current strategies directed at the inhibiting renin-angiotensin system, which may translate to an improvement in ventricular performance and survival. There have been significant advances in the pharmacological therapies used in chronic heart failure, with medication largely directed at inhibiting maladaptive neurohumoral responses.

8.2.2 ACUTE HEART FAILURE

As the prevalence of chronic heart failure rises, an accompanying rise in acute heart failure is predicable, along with the unavoidable accompanying morbidity, mortality and economic burden [Cowie *et al* 2000].

Designing appropriate studies in acute heart failure is not straightforward, and any study in this area will need careful consideration [McDonagh *et al* 2011]. Acute heart failure is a heterogeneous condition and may arise from decompensated, well-established chronic heart failure through to mechanical abnormalities such as ruptured mitral valve chordae; pharmacological intervention in the latter would be of questionable benefit. Furthermore, the benefit gained from short duration pharmacological intervention to treat a condition that has developed over a chronic duration is likely to be minimal.

Progress in acute heart failure has not shared the same advances in prognosis seen in chronic heart failure, and there has been little development with regard to new pharmacological agents [Konstam *et al* 2007; McMurray *et al* 2007; Miller *et al* 2008]. Furthermore, some studies report increased mortality [Sackner-Bernstein *et al* 2005] in this cohort when treated with agents that have a similar haemodynamic profile to apelin. One recent trial contradicts the main body of evidence and has reported favourable outcomes in patients presenting with acute heart failure treated with serelaxin [Teerlink *et al* 2013]. In addition to an improvement in initial symptoms, a signal of increased survival was observed. The trial was not powered to detected survival differences in each arm and this result is largely hypothesis generating; furthermore multiple tests were performed on these

data. Whilst reconciling the improved survival that result from a brief drug infusion, reduction in organ damage, as assessed by relevant circulating plasma biomarkers, was reported, which may offer some biological plausibility to the finding [Metra *et al* 2013].

There may be a potential role for apelin in the treatment of patients presenting with hypotension and impaired ventricular function, who need supportive therapy in order to increase ventricular function. The current inotropic therapies are arrhythmogenic and associated with adverse events. Whilst the inotropic action of apelin is perhaps modest, there is little change in mean arterial pressure such that initiation would be relatively straightforward and require minimal monitoring. We have limited data assessing the arrhythmogenic potential of APLNR agonism, with clinical trials limited to small numbers. Whilst we have used an infusion duration that would be suitable for acute heart failure treatment, larger numbers of trials are required to assess fully the safety of APLNR agonism.

The utility of apelin as a biomarker, in its current form, is limited. There are well-established biomarkers for heart failure, and at present the current commercially available apelin assays add little with respect to diagnosis or short term prognosis [van Kimmenade *et al* 2006]. Nonetheless, it is conceivable that more precise assays will become available and may prove to be more informative.

8.2.3 VASCULAR DISEASE

Atheroma

Preclinical data show that apelin inhibits angiotensin II-induced aneurysm formation and limits atheroma progression [Leeper *et al* 2009]. This effect is mediated through nitric oxide and is independent of blood pressure. The data from these studies are taken from ApoE knockout rodent models that represent an extreme phenotype not receiving evidenced-based lipid-lowering medication. These effects may not be present in humans, and furthermore an incremental benefit on current medical therapy would need to be proven. Again, any studies that wish to explore this area of research will require an oral preparation in order to assess these chronic effects.

Systemic Hypertension

APLNR signalling may be of benefit in systemic arterial hypertension. Preclinical and clinical studies have shown a reduction in mean arterial pressure, and whilst largely modest, this may be a useful adjuvant to current therapy. Whilst the best clinical outcomes in hypertensive patients are observed in those that experience the largest reduction in blood pressure, modest reductions in blood pressure still reduce the risk of heart failure and strokes [Czernichow *et al* 2011].

Idiopathic Pulmonary Arterial Hypertension

There are emerging data supporting a significant role for the apelin-APLNR system in pulmonary hypertension. Preclinical models of hypoxic- and monocrotaline-induced pulmonary hypertension show downregulation of the apelin-APLNR system in pulmonary hypertension [Falão-Pires *et al* 2009; Chandra *et al* 2011]. Rodents deficient in apelin and exposed to hypoxia develop more severe pulmonary hypertension than wild types, and small pulmonary arterioles are obliterated

[Chandra *et al* 2011], therefore suggesting that the apelin-APLNR system protects against the development of IPAH. Interestingly, treatment with apelin prevents the onset of monocrotaline-induced pulmonary hypertension [Falão-Pires *et al* 2009]. There are limited clinical data in patients in this cohort; however, the apelin-APLNR system does appear to contribute to the disease. In patients with pulmonary arterial hypertension a reduction in circulating plasma apelin has been identified [Goetze *et al* 2006].

Given the inotropic action of apelin and its downregulation in pulmonary hypertension, there may be some therapeutic potential for this hormone system. In humans, the majority of data regarding apelin and ventricular dysfunction come from studies primarily assessing left ventricular dysfunction. Brief systemic infusions increase cardiac output in healthy volunteers, and this effect is preserved in patients with chronic stable heart failure [Japp *et al* 2010]. Furthermore, in studies assessing the prolonged infusion of (Pyr^{1})apelin 13, efficacy is retained and there are no adverse effects. The inotropic action of apelin may be critical in IPAH, as right ventricular function predicts patient outcomes [van de Veerdonk *et al* 2011]; consequently, the effect of APLNR agonism on cardiopulmonary performance merits investigation in this cohort.

The precise physiological role of apelin, and hence the consequences of reduced plasma concentrations in IPAH, are unknown. Given the biological properties of apelin and the data from heart failure studies, it is likely that apelin-APLNR contributes to the pathophysiology of IPAH.

Increasingly, therapies that are effective in treating left ventricular failure are being investigated in patients with IPAH, as it is becoming evident that right ventricular function is the key predictor of survival in this cohort [de Man *et al* 2012]. A series of clinical studies has been planned to characterise the pulmonary and systemic cardiovascular response to apelin in pulmonary hypertension. We plan to investigate patients with idiopathic pulmonary arterial hypertension, pulmonary hypertension secondary to left heart disease and healthy control subjects. We will recruit 21 patients for each group and the subjects will attend for invasive haemodynamic studies. During right heart catheterisation, the patients will receive systemic (Pyr^{1})apelin-13 infusions at 3, 10 and 30 nmol/min for 10 minutes or a placebo in a double blinded crossover design.

We predict that apelin will increase cardiac output and reduce pulmonary arterial pressure, and as such the haemodynamic effects of apelin will be proven beneficial in pulmonary hypertension. This is the first human study designed to assess the vascular effect of apelin in the pulmonary circulation *in vivo.*

We have assessed the feasibility of apelin infusion during cardiopulmonary exercise testing, the results of which are presented above. Our next objective is to assess the effect of (Pyr^{1})apelin-13 on exercise in patients with IPAH. We aim to recruit 12 stable patients who have been on their current medication for at least 8 weeks and will attend up to three occasions at least one week apart for cardiopulmonary exercise testing; patients that have a baseline cardiopulmonary exercise test within

4 weeks of entry will not be required to undertake a baseline test. On the first visit, the participants will undertake a cardiopulmonary exercise by completing a ramp incremental exercise test to establish VO_{2PEAK}. Thereafter, they will attend two further exercise tests, exercise at 80% of their maximal workload and be randomised in a double blinded crossover design to either (Pyr^{1})apelin-13 or a placebo. We predict that (Pyr^{1})apelin-13 will increase exercise ability in patients with IPAH.

Perhaps idiopathic pulmonary arterial hypertension provides a unique opportunity to circumvent the current pharmacokinetic limitations of apelin. Some of this patient population are treated with intravenous therapies through indwelling Hickman lines, and, provided that combining these preparations in solution is safe, there is potential to administer apelin for long durations. In addition to the haemodynamic effects of apelin, this would allow for an investigation of chronic dosing and any effects of ventricular and vascular remodelling.

8.3 CONCLUDING REMARKS

The apelin-APLNR system is in its infancy. Further work is necessary to fully characterise this system and understand its role in cardiovascular physiology and pathophysiology. Most obviously, a reliable assay that reliably detects plasma apelin concentration would allow definite understanding of plasma concentrations in patients with cardiovascular disease. Additionally such an assay would assist in understanding pharmacokinetic properties of exogenous apelin, which may refine dosing regimes and help explain current dose response data.

No reliable antagonist is available. The ability to inhibit APLNR signalling would greatly enhance understanding the contribution of the apelin-APLNR to myocardial performance under basal and resting conditions.

In the studies presented in this thesis we have demonstrated that the efficacy of the apelin-APLRN system is retained during conditions of renin-angiotensin activation. Furthermore in healthy and diseased myocardium there is sustained response to APLNR activation. Taken together these studies suggest that this system is a viable target in cardiovascular disease and merits further investigation.

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APPENDIX

SODIUM RESTRICTED DIET PLAN

Check nutritional information on food labelling just in case

In general any processed food or constituent will contain high levels of salt and must be avoided during the salt restricted diet. Most condiments will be high in salt – if there is any doubt please avoid foods that you think may contain salt.

Drinks

- Water
- Black Tea (only a dash of milk) – NB: not 24hours before study
- Herbal teas
- Instant/Black Coffee (only a dash of milk) – NB: not 24hours before study
- Cranberry /orange/grapefruit /tropical/apple juice
- 40%/47.5% spirits – Nb: not 24 hours before study
- *No carbonated drinks*

Food

Breakfast

- Shredded wheat
- Bitesize shredded wheat
- Sugar puffs
- Kelloggs Frosted Wheats

NB: No milk – could try baby/formula milk or juice?

- Porridge - Porridge oats/oatmeal with water

Carbohydrates

- Brown/white Rice
- Spaghetti/macaroni/pasta **(Do not use salt)**
- Barley
- Cous cous
- Potatoes – boiled, roasted, jacket

Oils, condiments, seasonings

- Sunflower oil/olive oil/vegetable oil/rapeseed oil
- Mint/pepper/oregano/mustard seeds/sage/ mixed herbs/tarragon/basil/ginger/cinnamon
- Red/white wine vinegar
- Home made dressing, fresh lime juice with olive oil - no salt

Snacks

- Cooking chocolate - Silver spoon – milk chocolate chips
- Snack a Jacks
    - Berry flavour
    - popcorn – chocolate flavour
- Plain unsalted popcorn
- Sunflower seeds
- Brazil nuts
- Almonds
- Pine nuts
- Pumpkin seeds

Please check to make sure that there is no salt added to nuts

Fruit

- Apples
- Pears
- Peaches/nectarines/apricot
- Oranges/satsumas/mandarins/clementines
- Plums
- Kiwi
- Strawberries /raspberries /cherries/blackberries/cranberries
- Grapes
- Lemon/lime
- Pomegranate
- Pineapple
- avocado

Fresh Vegetables

Nb: if boiling –do not use salt

- beansprouts
- green beans/mangetout/sugar snap peas/ garden peas/ petit-pois
- soya beans
- chickpeas
- lettuce
- cabbage
- cucumber
- broccoli
- mushrooms
- baby corn
- sprouts
- onions/spring onions
- carrots
- leeks

- peppers
- parsnip
- tomatoes
- courgette
- garlic
- Potatoes – boiled, roasted, jacket

Meats

- chicken/turkey breast - boiled/roasted/grilled/casseroled
- lamb shoulder - roasted/stewed/braised
- Rack of lamb – roasted
- Lamb mince – stewed
- Lamb loin chop – grilled / roasted
- Pork loin chop - roasted
- Tofu

Avoid stock cubes and adding salt when cooking

Sample Meal Plan

Breakfast

- *See breakfast above*
- Fruit

Lunch

- Grilled Chicken Pasta Salad
    - Lettuce, carrot, mushrooms, cucumber, spring onions, red pepper, avocado, tomatoes etc. with chicken and pasta with a white wine vinegar
- Jacket potato and salad

Snacks

- *See snacks above*
- Fruit
- Carrots/peppers/celery sticks
- Rice cakes

Dinner

- Grilled Chicken with stir fry vegetables (e.g. baby corn, courgettes, peppers, tomatoes, beansprouts, broccoli etc) and boiled rice/spaghetti/ plain cous cous

- Chicken fried rice
    - E.g. Chicken, peas, peppers, carrots, broccoli, aubergine

    - Fried with a little oil – No soya sauce!

- Chicken with chilli and coriander drizzled with olive oil with boiled potatoes and vegetables
- Roasted Turkey/chicken breast with cranberry sauce with roasted potato and vegetables
- Grilled portabello mushroom with chilli with boiled potatoes and salad

The most simple way to keep to this diet will be to have a fruit based breakfast, and grilled meats (from above) with boiled veg or salad – avoiding any salt or butter.

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